Functional EF-Tu with large C-terminal extensions in an *E. coli* strain with a precise deletion of both chromosomal *tuf* genes

Robert Schnell[a,b], Farhad Abdulkarim[a], Miklós Kálmán[b], Leif A. Isaksson[a,*]

[a]*Department of Microbiology, Stockholm University, S-106 91 Stockholm, Sweden*
[b]*Bay Zoltán Foundation for Applied Research, Institute of Biotechnology, Szeged, Hungary*

Abstract An ***Escherichia coli*** **strain was constructed in which both chromosomal genes encoding elongation factor (EF)-Tu (*tufA* and *tufB*) have been inactivated with precise coding sequence replacements. A *tufA* gene in an expression vector is supplied as the sole EF-Tu source. By using plasmid replacement, based on plasmid incompatibility, mutant EF-Tu variants with a large C′-terminal extension up to 270 amino acids were studied and proved to be functional in a strain lacking the chromosomal *tufA* and *tufB* genes.**
© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Elongation factor; Protein fusion; Plasmid exchange; Gene replacement

1. Introduction

Elongation factor (EF)-Tu plays a central role in the prokaryotic translational apparatus by supplying the ribosomal A site with charged tRNA during peptide chain elongation [1]. The gene encoding this translation factor is present in multiple copies in the genome of many organisms [2]. Two EF-Tu genes (*tuf*) are encoded in the genomes of *Escherichia coli* [3] and *Salmonella typhimurium* [4], probably reflecting a gene duplication event [1]. The multiple copies of the gene and the fact that both genes are members of operons with other indispensable genes make it difficult to use the arsenal of bacterial genetics to study mutant variants of this important translation factor.

Several approaches have been taken to inactivate either one of the encoding genes in *E. coli*, *tufA* or *tufB*. Insertional inactivation of *tufB* could be achieved easily [5]. However, *tufA* could not be inactivated by a similar approach [6]. The two proteins differ only in the last encoded amino acid residue – glycine in *tufA* and serine in *tufB*. The operon organization shows even more differences: *tufA* is encoded as the last gene in the *rpsL* operon containing the genes *rpsL-rpsG-fusA-tufA* at the 74.7 min map position [7], whereas *tufB* is encoded in an operon with the *thrU-tyrU-glyT-thrT-tufB* genes at the 89 min map position [8], also in this case being the last gene in the operon. Functional differences between the two EF-Tu species have not been observed [6,9]. Explanations for the unsuccessful attempts to inactivate the *tufA* gene have been

attributed to the importance of the other genes encoded in the same operon, or to the possible secondary effect of the sequence alterations or transposon insertions. Alternatively, the higher expression level of the *tufA* gene might not be compensated by a single *tufB* copy. Nevertheless, Kraal and coworkers later showed that, using precise coding sequence replacement, either one of the *tuf* genes could be deleted, making it possible to characterize the physiological effects caused by inactivation of either one of the two *tuf* genes [10]. Successful inactivation of both chromosomal *tuf* genes in the same strain has not yet been reported.

In the present work, we have inactivated both chromosomal *tuf* genes by replacing *tufB* with a kanamycin phosphotransferase cassette ($\Delta tufB::kan$), and *tufA* with a chloramphenicol acetyltransferase cassette ($\Delta tufA::cat$). The sole EF-Tu source is a plasmid-encoded *tufA* gene in an expression vector, under control of the P_{trc} promoter and a *Lac*I-O_{lac} system. Due to the high level of EF-Tu needed, expression induction by IPTG is necessary for growth, making the double deletion strain IPTG dependent. In order to construct strains with different mutant EF-Tu variants and make it possible to study these proteins and their functional influences in the absence of any chromosomal EF-Tu contribution, a plasmid replacement system was used. By using this plasmid replacement procedure, plasmids encoding mutant EF-Tu variants with C′-terminally tagged proteins were introduced as the sole source of EF-Tu. As judged by physiological properties such as growth rate, IPTG dependence, and kirromycin sensitivity, the C′-terminally tagged EF-Tu with an extension up to 323 amino acids is functional.

2. Materials and methods

2.1. E. coli *strains and media*

The K12 *E. coli* strains used in this study are MG1655 (*tufA*[+], *tufB*[+]), UI232 (*tufA*(Kir-R), *tufB*(B_o)) [11], and DH5α. The media were made as described by Miller [12]. The strains were grown in LB broth, if nothing else is stated. To select for antibiotic resistance, 100 μg/ml ampicillin, 25 μg/ml tetracycline, 30 μg/ml kanamycin or 50 μg/ml kirromycin was used. IPTG was used in the 0.1–1.0 mM concentration range to induce expression of the cloned genes.

2.2. PCR amplifications

The gene encoding EF-Tu (*tufA*) was obtained by using the PCR procedure and a chromosomal template from the strain MG1655. The reaction mixtures were made for Vent polymerase (NEB) following the manufacturer's protocol, with minor modifications as indicated in the text. To amplify the *tufA* gene including its stop codon, the oligonucleotide SR-7 (5′-GCTGAATT<u>CGTGTCTAAAGAAAAATTT-GAACG</u>-3′; sequence homology with the template is underlined) was used as upstream primer and SR-8 (5′-GTCGGATC<u>CGCCCTTTT-

*Corresponding author. Fax: (46)-8-6129552; www.mibi.su.se.
E-mail address: leif.isaksson@mibi.su.se (L.A. Isaksson).

CAATTCAAAAC-3′; sequence homology with the template is underlined) as downstream primer. For C′ fusion of the *tufA* gene, SR-7 was used as upstream primer and SR-9 (5′-ATAGGATC<u>CGCCC-GAAACTTTAGCAACAAC</u>-3′) as downstream primer. The reaction mixture with 2 mM $MgCl_2$ was pre-heated to 95°C for 2.5 min; thereafter, 30 cycles were run with denaturation for 60 s at 94°C, annealing for 60 s at 55°C and extension for 2 min at 72°C. After the cycles were completed, further incubation at 72°C for 5 min was used as terminal extension step.

2.3. Plasmid constructions

All plasmids described in this study are illustrated schematically in Fig. 1. DNA work was performed as described by Sambrook et al. [13]. Restriction enzymes were obtained from New England Biolabs, Gibco Life Sciences, and MBI Fermentas. DNA fragments were isolated using Qiaex II Gel Extraction Kit (Qiagen). Plasmid preparations were done using Qiagen and Jetprep plasmid preparation kits.

PCR-amplified tufA sequences with or without the original stop codon were cloned into pTRC99A (Pharmacia Biotech), using flanking upstream *Eco*RI and downstream *Bam*HI restriction sites, resulting in the plasmids pTRCtufA and pTRCtufACEX, respectively. The tetracycline-resistant variant of pTRCtufACEX is pTRCtufACEX-Tc, which confers tetracycline but not ampicillin resistance. To construct the latter plasmid, the unique *Fsp*I site within the *bla* gene was used to insert an *Eco*RI–*Sty*I fragment of pBR322 encoding the *tet* gene with blunt ends filled in with Vent-polymerase.

In order to construct C′-terminal fusions to EF-Tu, a filled-in downstream *Bam*HI site in pTRCtufACEX was used to join the *tufA* coding sequence to a blunt upstream end of Z domain multimer-coding fragments generated by *Fsp*I cleavage. This gave an in-frame coding sequence fusion and a protein linker sequence of 7 amino acid residues (GSQHDEA) between the two fused proteins. *Hin*dIII sites at the downstream end of the Z multimer fragments were used to construct tufA-Z_2, tufA-Z_3, tufA-Z_4, and tufA-Z_5 gene fusions in plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 and pTRCtufAZ5, respectively.

2.4. Preparation of fusion proteins

Purification of proteins on IgG-Sepharose (Amersham-Pharmacia Biotech) was performed as described by Nilsson et al. [14]. All samples were lyophilized.

2.5. Protein gel electrophoresis

SDS–polyacrylamide gel electrophoresis (PAGE) was used for protein separation. The gels (0.75 mm thick) were run in a Pharmacia Midget gel apparatus with 5% (w/v) polyacrylamide as stacking and 15% (w/v) as resolution gels. Protein bands were visualized by Coomassie brilliant blue G staining [15].

2.6. Doubling time determinations

Log phase cultures with bacteria were grown in LB medium supplemented with ampicillin (100 µg/ml) and IPTG (1 mM); growth was followed at 37°C by measuring the OD_{540}.

2.7. Gene deletions

Strains constructed in this study are shown in Table 1. Chromosomal inactivation of the *tufB* gene in *E. coli* MG1655 was completed by homologous recombination using the pKNG101-mediated gene deletion system [16]. The coding sequence of *tufB*, including the start codon ATG and the stop signal TAA, was replaced with the kanamycin phosphotransferase gene of the plasmid pUC4K (Pharmacia) with flanking *Eco*RI sites, resulting in the strain SR92 (Δ*tufB*::*kan*). Strain AZ32 (Δ*tufA*::*cat*) was obtained from B. Kraal [10]. Using SR92 as a host, with either plasmids pTRCtufA or pTRCtufACEX, it was possible to remove the *tufA*+ gene by P1 phage transduction [17] using a lysate originating from AZ32 (*tufA*::*cat*) [10]. As a result, strains were obtained having both chromosomal *tuf* genes deleted (*tufA*::*cat* (74.7 min); *tufB*::*kan* (89.9 min)) and harboring the plasmids encoding different variants of EF-Tu.

2.8. Plasmid replacement

Starting with strain SR143 [*tufA*::*cat*; *tufB*::*kan*] the plasmid pTRCtufACEX expressing EF-Tu could be replaced by plasmids encoding modified EF-Tu genes. Competent cells were prepared and transformations were carried out as described by Inoune et al. [18], but 3–4 h long phenotypic expression was necessary for successful transformation, before spreading the cells on the selective plates. SR143, harboring the plasmid pTRCtufACEX, was transformed with the plasmid pTRCtufACEX-Tc and plated on tetracycline/IPTG plates to select for the latter plasmid. The transformants were screened for ampicillin sensitivity to indicate the loss of pTRCtufACEX. Next, SR143 cells harboring the plasmid pTRCtufACEX-Tc were transformed with plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 or pTRCtufAZ5 conferring resistance to ampicillin. Transformants were selected on ampicillin/IPTG plates and screened for tetracycline sensitivity to indicate the loss of pTRCtufACEX-Tc.

3. Results

An *E. coli* strain was constructed in which both chromosomal genes encoding EF-Tu have been precisely replaced by antibiotic resistance cassettes (Δ*tufB*::*kan*;Δ*tufA*::*cat*). The sole EF-Tu source in the cell is provided by a plasmid-encoded *tufA* gene. The plasmid expressing EF-Tu was constructed to give resistance to either ampicillin or tetracycline. Such plasmids can be replaced with each other as a result of plasmid incompatibility, as demonstrated for plasmids with the same replication origin and different resistance markers. Using this plasmid-replacement procedure, plasmids encoding mutant EF-Tu variants with C′-terminally tagged proteins can be introduced as the sole sources of EF-Tu.

3.1. Construction and functional test of C′-terminally tagged EF-Tu variants

The coding sequence of *tufA*+ with its original stop codon was cloned by PCR and ligated into pTRC99A (Pharmacia-Biotech), resulting in the plasmid pTRCtufA. In this expres-
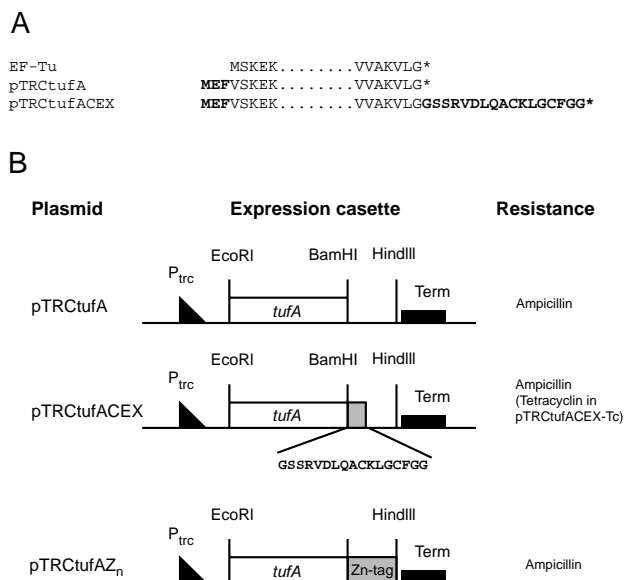

Fig. 1. A: Sequence alterations in studied EF-Tu protein variants. The addition of three N′-terminal amino acids (MEF) and the replacement of the N′-terminal methionine (GUG) with valine are due to sequence specificities in the expression vector in plasmids pTRCtufA and pTRCtufACEX, and the C′-tagged variants. The amino acid sequence of the short C′-terminal extension tag is also shown for pTRCtufACEX. B: Expression cassettes in the plasmids showing the used restriction enzyme sites, the Trc promoter (P_{trc}), and the $rrnB T_1 T_2$ terminator (Term). The Z_n tag stands for the C′-terminal extensions consisting of 2, 3, 4 or 5 IgG binding Z domains in plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 or pTRCtufAZ5, respectively.

Table 1
Doubling times for strains with different plasmid-encoded EF-Tu variants and the effect of inactivation of either or both *tuf* genes

Strain	Chromosomal genotype	Plasmid	Doubling time (min)
MG1655	*tufA*+, *tufB*+	pTRC99A	32
SR92	*tufB::kan*	pTRC99A	35
AZ32	*tufA::cat*	pTRC99A	44
SR143	*tufA::cat*, *tufB::kan*	pTRCtufA	59
SR143	*tufA::cat*, *tufB::kan*	pTRCtufACEX	72
SR143	*tufA::cat*, *tufB::kan*	PTRCtufAZ2	109
SR143	*tufA::cat*, *tufB::kan*	PTRCtufAZ3	109
SR143	*tufA::cat*, *tufB::kan*	PTRCtufAZ4	147
SR143	*tufA::cat*, *tufB::kan*	pTRCtufAZ5	100

Doubling time values were determined for strains with inactivated chromosomal *tuf* genes (SR143) with plasmids expressing wild-type EF-Tu or C′-terminally tagged variants, as indicated.

sion plasmid, the *tufA* gene is under control of the P_{trc} promoter and a *Lac*I-O$_{lac}$ system (Fig. 1).

To construct *tufA* with C′-terminal fusions, the *tufA* coding sequence without the original stop codon was cloned by PCR and ligated into pTRC99A expression vector, resulting in the plasmid pTRCtufACEX. The expression is under the control of the P_{trc} promoter and the *Lac*I-O$_{lac}$ system, and the expressed protein has a 17 amino acid long C′-terminal sequence tag (GSSRVDLQACKLGCFGG) (Fig. 1A). The gene also carries a set of unique restriction enzyme recognition sites in the coding sequence of this short tag, making it possible to insert heterologous sequences in-frame with the *tufA* coding sequence, thus giving different C′-terminal protein fusions. A tetracycline-resistant variant (pTRCtufACEX-Tc) of pTRCtufACEX was also constructed that confers tetracycline but not ampicillin resistance after this modification.

In order to construct 3′-terminal fusions to *tufA*, IgG binding Z domain multimer-coding fragments (Z$_2$, Z$_3$, Z$_4$, and Z$_5$) were cloned into pTRCtufACEX, resulting in *tufA*-Z$_2$, *tufA*-Z$_3$, and *tufA*-Z$_4$, and *tufA*-Z$_5$ gene fusions in plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 and pTRCtufAZ5, respectively. The sequence between the fused genes codes for a protein linker of seven amino acid residues (GSQHDEA).

In the *E. coli* strain UI232, *tufA* is mutated to give a kirromycin-resistant EF-Tu (A$_r$) while *tufB* is mutated to give a non-functional protein product (B$_o$) [11], making the strain

kirromycin-resistant. This phenotype has previously been shown to be overruled by introducing a plasmid-borne wild-type *tufA*+ gene that expresses a kirromycin-sensitive EF-Tu [19,20]. To determine if the kirromycin-resistant phenotype of strain UI232 is complemented by the fusion protein constructs, this strain was transformed with plasmids pTRC99A, pTRCtufA and pTRCtufACEX, and by the constructs pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 and pTRCtufAZ5 which express the fusion proteins. The transformants were tested for growth on Ap, Ap/IPTG, Ap/Kir, and Ap/IPTG/ Kir plates. As shown in Table 2, the control strain UI232 with plasmid pTRC99A grows as expected on all four test plates. UI232 transformed with pTRCtufA or pTRCtufACEX grows on Ap, Ap/IPTG, and Ap/Kir plates, but not on Ap/IPTG/ Kir plates. The test of plasmid pTRCtufACEX-Tc and the plasmids encoding the C′-terminally tagged fusion proteins gave similar results. These findings suggest that the plasmid-borne EF-Tu proteins are functional, since they change the phenotype from kirromycin resistance to sensitivity upon expression.

3.2. Gene deletions

Using chromosomal gene replacement [16], the coding sequence of the chromosomal *tufB* gene was replaced with a kanamycin resistance cassette from pUC4K (Pharmacia), resulting in the strain SR92. With SR92 as a host, in the presence of either pTRCtufA or pTRCtufACEX, it was possible to remove the chromosomal *tufA*+ gene using P1 phage transduction with a lysate originating from strain AZ32 (*tufA::cat*) [10]. These steps gave the strain SR143 that has both chromosomal *tuf* genes deleted [*tufA::cat* (74.7 min); *tufB::kan* (89.9 min)] (Fig. 2). Due to the high level of EF-Tu needed,
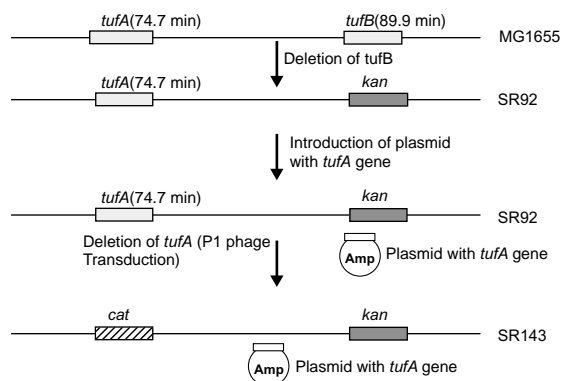

Fig. 2. Inactivation of both chromosomal *tuf* genes. The *tufB* gene was replaced with a kanamycin phosphotransferase cassette (*kan*) by pKNG101-mediated chromosomal gene replacement, resulting in the strain SR92(*tufB::kan*). Next, in the presence of plasmids expressing EF-Tu (pTRCtufA and pTRCtufACEX), the chromosomal *tufA* gene was replaced with a chloramphenicol acetyltransferase cassette (*cat*) by P1 phage transduction using a lysate originating from AZ32 (*tufA::cat*). The resulting strain was SR143 (*tufA::cat*;*tufB::kan*) with either plasmid pTRCtufA or pTRCtufACEX.

Table 2
Kirromycin sensitivity test for plasmid-expressed EF-Tu and C′-terminally tagged variants in strain UI232 (A$_r$, B$_o$)

Plasmid	Amp	Amp, IPTG	Amp, Kir	Amp, Kir, IPTG
pTRC99A	+	+	+	+
pTRCtufA	+	+	+	−
pTRCtufACEX	+	+	+	−
pTRCtufAZ2	+	+	+	−
pTRCtufAZ3	+	+	+	−
pTRCtufAZ4	+	+	+	−
pTRCtufAZ5	+	+	+	−

Growth of strain UI232 with different plasmids was tested on different plates, as indicated. The change from kirromycin resistance to sensitivity upon expression of the indicated EF-Tu variants including the C′-terminally tagged constructs EF-TuCEX, EF-Tu-Z$_2$, EF-Tu-Z$_3$, EF-Tu-Z$_4$ or EF-Tu-Z$_5$ is shown. Amp, ampicillin; Kir, kirromycin.
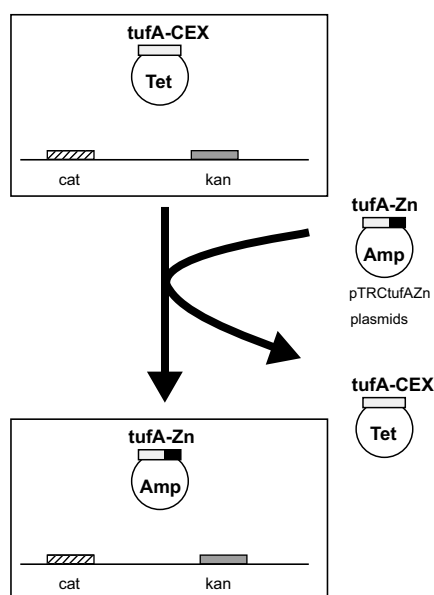
Fig. 3. Plasmid replacement technique based on plasmid incompatibility. The indicated EF-Tu-expressing plasmids were exchanged in strain SR143 (*tufA*::*cat*;*tufB*::*kan*). As a result, plasmids expressing C′-terminally tagged EF-Tu variants are the sole sources of EF-Tu.

the presence of IPTG is necessary for growth. The double deletion strain is IPTG dependent, and the doubling time values showed no significant difference when IPTG was used at 0.1 mM or 1.0 mM concentrations in the case of constructs pTRCtufA and pTRCtufACEX. The higher IPTG concentration gave significantly faster growth in case of the C′-terminally tagged constructs pTRCtufAZ2 and pTRCtufAZ3 (data not shown). This indicates that even though the fused protein is functional, the C′-terminal extensions decrease the efficiency of the EF-Tu cycle.

3.3. The plasmid replacement system

In the strain SR143, in which both *tuf* genes are inactivated, plasmids encoding different EF-Tu variants could be exchanged by using plasmid incompatibility. To do this, SR143 harboring the plasmid pTRCtufACEX was transformed with the plasmid pTRCtufACEX-Tc and plated on tetracycline/IPTG plates. Tetracycline-resistant transformants were screened for ampicillin sensitivity. The resulting Amp^{S}Tet^R colonies harboring only the latter plasmid were tested in further steps for kanamycin resistance, chloramphenicol resistance and IPTG dependence to confirm the chromosomal genotype and dependence on the plasmid-encoded *tuf* gene. In this way, clones were isolated that harbor the plasmid pTRCtufACEX-Tc instead of its ampicillin-resistant version pTRCtufACEX, maintaining the chromosomal background as in SR143.

The duration of the phenotypic expression step in these plasmid replacement steps had to be 3–4 h long. The necessary length of the phenotypic expression step can be correlated with the three- to four-fold longer doubling time of these strains (see also Section 2). Finally, cells were transformed with the plasmid set encoding the engineered EF-Tu variants pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 or pTRCtufAZ5. The transformants were selected on ampicillin/IPTG plates and screened for tetracycline sensitivity; this identifies clones that have lost the shuttle plasmid but harbor the newly intro-

duced plasmids encoding the C′-tagged EF-Tu fusion proteins (Fig. 3). The Amp^{R}Tet^S colonies were tested in further steps for kanamycin resistance, chloramphenicol resistance and IPTG dependence phenotype. The kirromycin sensitivity of these constructs was confirmed, and the expression of the corresponding modified EF-Tu proteins were checked by affinity purification and gel electrophoresis (not shown).

3.4. Growth effects by tuf-gene inactivation

The doubling time values of the constructed strains [*tufA*:: *cat* (74.7 min); *tufB*::*kan* (89.9 min)] with plasmids encoding EF-Tu or C′-tagged EF-Tu proteins were used to compare the effect on growth of the engineered EF-Tu variants and their wild-type counterpart. MG1655 with plasmid pTRC99A was used as wild-type control. SR92[*tufB*::*kan*] with pTRC99A and AZ32[*tufA*::*cat*] with pTRC99A were also used as controls to identify the effect of either *tuf*A or *tuf*B inactivation.

Derivatives of SR143 [*tufA*::*cat*;*tufB*::*kan*] with plasmids pTRCtufA, pTRCtufACEX, pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 or pTRCtufAZ5 were also analyzed. The doubling time values obtained in this experiment (Table 1) suggest that even though the strains are viable, the C′-terminal extensions have a negative effect on EF-Tu function. This negative effect is correlated with the size of the C′-terminal tag. However, in the case of the longest extension, cultures tend to grow faster as a result of recombinational loss of Z domain fusion tags, as observed analyzing affinity-purified fusion proteins and plasmids from these constructions (not shown).

3.5. Analysis of EF-Tu-Z$_n$ proteins

The presence and stability of the EF-Tu-Z fusion proteins in different strains was investigated by purifying these proteins on IgG-Sepharose affinity columns. A high yield of protein was eluted from columns with samples from strain UI232 with pTRCtufAZ$_n$ plasmids. As expected, no significant amount of protein material was recovered from the affinity column for
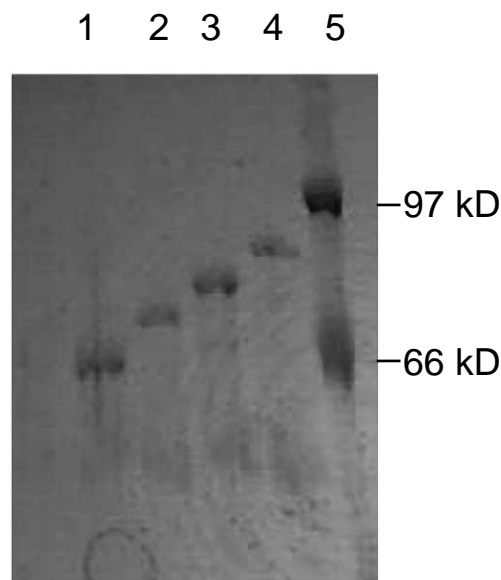

Fig. 4. SDS–PAGE analysis of C′-terminally tagged EF-Tu proteins purified on IgG affinity columns. Lane 1: EF-Tu-Z$_2$, lane 2: EF-Tu-Z$_3$, lane 3: EF-Tu-Z$_4$, lane 4: EF-Tu-Z$_5$, lane 5: molecular weight marker; bands corresponding to 66 and 97 kDa are indicated.

the control strain (UI232/pTRC99A). Samples of the IgG-Sepharose-purified material were analyzed using SDS–PAGE. As can be seen in Fig. 4, the mobility of the protein bands suggests a molecular weight that corresponds to the expected sizes of the fusion proteins 60.1, 66.7, 73.2 and 79.8 kDa for EF-Tu-Z_2, EF-Tu-Z_3, EF-Tu-Z_4 and EF-Tu-Z_5, respectively (Fig. 4).

The fusion proteins were also isolated from strain SR143 ($tufA$::cat; $tufB$::kan) harboring the plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 and pTRCtufAZ5. The EF-Tu-Z_2 and EF-Tu-Z_3 protein bands were homogeneous on SDS gels, indicating that these constitutions are stable. In the case of the two longest extensions, -Z_4 and -Z_5, the proteins isolated from strain SR143 were not homogeneous. The observed shortening of the fusion tag appears to be the result of plasmid recombination, as evidenced by analysis of plasmid preparations from this strain (not shown).

Total cell protein samples from wild-type E. coli MG1655, from the derived double deletion strain SR123/pTRCtufA harboring a plasmid expressing wild-type EF-Tu, and from SR123/pTRCtufAZ2 expressing a C′-terminally tagged EF-Tu with 11 kDa higher molecular weight, were analyzed on SDS gels (Fig. 5). The Coomassie-stained protein gel revealed that the characteristic EF-Tu band (43 kDa) that is present in the wild-type E. coli total protein sample was replaced by a very prominent band (43 kDa) in the case of the SR123/pTRCtufA sample. This reflects the over-expression of EF-Tu from the plasmid. The cells that had both chromosomal tuf genes deleted and expressed C′-terminally tagged EF-Tu from pTRCtufAZ2 as sole EF-Tu source, lacked the charac-

teristic EF-Tu band in the 43 kDa molecular weight range. Instead, they gave a strong band corresponding to an EF-Tu-Z_2 over-expressed fusion protein in the expected 60 kDa size range (Fig. 5).

4. Discussion

The IgG binding Z domain consists of 54 amino acid residues (excluding extra sequences) [14], resulting in fusion tags of 149, 207, 265, and 323 amino acid residues in length (including linker and additional C′-terminal sequences). The finding that the analyzed fusion proteins can support the complex functional repertoire of EF-Tu in growing cells indicates that the C′-terminus is probably not directly involved in the cell in any essential binding interactions with other proteins or complexes such as EF-Ts, tRNAs or the ribosome. These engineered proteins therefore can be exploited in structural analysis of the ribosome in its kirromycin-blocked state, serving as a physical reporter of the ribosome-bound EF-Tu.

Crystal structures of EF-Tu in complex with GTP and tRNA [21] and the GTP–GDP exchange factor EF-Ts [22] show that the C′-terminus is not part of the contact surface of EF-Tu with these molecules. However, the N′-terminus is probably involved in the ribosome-docking surface. Fitting the components of the ternary complex into the cryo-electron microscopy density-map of kirromycin-blocked ribosomes [23] suggests that although the C′-terminal end of EF-Tu does not take part in ribosomal interactions, this is not true for the N′-terminal end. This observation is in line with our results: N′-terminally tagged EF-Tu molecules proved to be defective in function (not shown), suggesting the possible role of the N′-terminal domain in the interactions mentioned above. However, a three amino acid residues long extension in the amino-terminus does not interfere with EF-Tu function (Fig. 1, Table 2).

Due to the high level of EF-Tu needed in the cell, induction of the plasmid-borne tuf gene is necessary for growth when the sole EF-Tu source is a plasmid-encoded gene. Such IPTG dependence is a helpful tool to analyze the plasmid and plasmid-encoded tuf gene dependence of the $tufA$- and $tufB$-deleted strains. The doubling time values showed no significant difference when IPTG was used at 0.1 mM or 1.0 mM concentrations in the case of constructs pTRCtufA and pTRCtufA-CEX. However, the higher IPTG concentration gave significantly faster growth in case of the C′-terminally tagged constructs pTRCtufAZ2 and pTRCtufAZ3 (data not shown). This indicates that even though the fused protein is functional, the C′-terminal extensions decrease the efficiency of the EF-Tu cycle.

The C′-terminal extensions do have a negative effect on EF-Tu function, because the growth rate is decreased. This negative effect on growth rate is correlated with the size of the C′-terminal tag. However in the case of the longest extension, the negative effect is strong enough to provide a selection pressure for the recombinational loss of Z domain fusion tags. The duration of the phenotypic expression step in the plasmid replacement steps had to be 3–4 h long. Based on our experience, this might have been the reason why it appeared to be difficult to construct chromosomal deletions for both $tufA$ and $tufB$ in the same strain. The necessary length of this extended phenotypic expression step can be correlated with the three- to four-fold longer doubling time of these
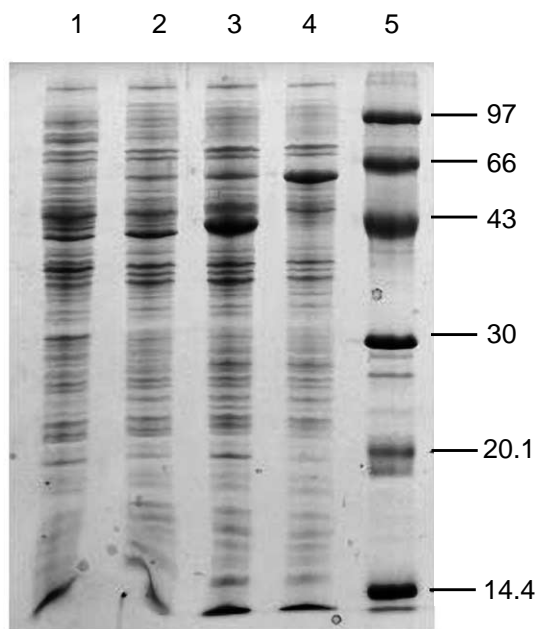

Fig. 5. SDS–PAGE analysis of EF-Tu in total protein samples. Proteins originating from strain MG1655/pTRC99A (lane 1), SR143/pTRCtufA induced with 0.1 mM IPTG (lane 2), SR143/pTRCtufA induced with 1.0 mM IPTG (lane 3) and SR143/pTRCtufAZ2 induced with 1.0 mM IPTG (lane 4). The molecular weight markers are indicated in kDa (lane 5). The characteristic band corresponding to EF-Tu appears in the 43 kDa molecular weight range in the samples analyzed in lanes 1–3. The prominent band with approximately 60 kDa molecular weight in lane 4 corresponds to EF-Tu-Z_2, which replaces the wild-type EF-Tu.

strains (see also Section 2). The double deletion strain expressed undoubtedly more EF-Tu from the plasmid than does the wild-type *E. coli* from the *tufA⁺* and *tufB⁺* genes in the chromosome (Fig. 5).

The results described above indicate that in a *tufB*::*kan*; *tufA*::*cat* background the demonstrated plasmid replacement technique using the pTRCtufaCEX-Tc as shuttle plasmid makes it possible to exchange plasmid-encoded EF-Tu variants. Thus, the genetic tools and strains used in this study enable the analysis of EF-Tu mutants and to illustrate possible EF-Tu-A/EF-Tu-B functional differences in vivo in the strain SR143 lacking chromosomal *tuf* genes ($\Delta tufB$::*kan*; $\Delta tufA$::*cat*).

References

[1] Krab, I.M. and Parmeggiani, A. (1998) Biochim. Biophys. Acta 1443, 1–22.

[2] Lathe, W.C. and Bork, P. (2001) FEBS Lett. 502, 113–116.

[3] Jaskunas, S.R., Lindhal, L., Nomura, M. and Burges, R. (1975) Nature 275, 458–462.

[4] Furano, A.V. (1978) Proc. Natl. Acad. Sci. USA 72, 4780–4784.

[5] Van de Klundert, J.A.M., Van der Miede, P.H. and Van der Putte Bosch, L. (1978) Proc. Natl. Acad. Sci. USA 75, 4470–4473.

[6] Vijgenboom, E. and Bosch, L. (1987) Biochimie 69, 1021–1030.

[7] Lindhal, L., Post, L., Zengel, J., Gilbert, S.F., Stychartz, W.A. and Nomura, M. (1977) J. Mol. Biol. 252, 7365–7383.

[8] Lee, J.S., An, G., Friesen, J.D. and Fiil, N. (1981) Cell 25, 251–258.

[9] Miller, D.L., Nagerkattis, S., Laursen, R.A., Parker, J. and Friesen, J.D. (1978) Mol. Gen. Genet. 159, 57–62.

[10] Zurmond, A.M., Rundölf, A.-K. and Kraal, B. (1999) Mol. Gen. Genet. 260, 603–607.

[11] Swart, G.W.M., Permaggiani, A., Kraal, B. and Bosch, L. (1987) Biochemistry 26, 2047–2054.

[12] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[14] Nilsson, B., Moks, T., Jansson, B., Abrahamsén, L., Elenstad, A., Holmgren, E., Henrichson, C., Jones, T.A. and Uhlén, M. (1987) Protein Eng. 1, 107–113.

[15] Neuhoff, V., Arnold, N., Taube, D. and Erhardt, W. (1988) Electroforesis 9, 255–262.

[16] Kaniga, K. and Delor, I. (1991) Gene 109, 137–141.

[17] Miller, J.H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[18] Inoue, H., Nojima, H. and Okayama, H. (1990) Gene 96, 23–28.

[19] Van de Klundert, J.A.M., den Turk, E., Borman, A.H., Van der Miede, P.H. and Bosch, L. (1977) FEBS Lett. 81, 303–307.

[20] Mesters, J.R., Zeef, L.A., Hilgenfeld, R., de Graaf, J.M., Kraal, B. and Bosch, L. (1994) EMBO J. 13, 4877–4885.

[21] Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Resketnikova, L., Clark, B.F. and Nyborg, J. (1995) Science 270, 1464–1472.

[22] Kawashima, T., Berthet-Colominas, C., Wulff, M., Cusack, S. and Lebermann, R. (1996) Nature 379, 511–518.

[23] Valle, M., Sengtupa, J., Swami, N.K., Grassucci, R., Burkhart, N., Neirhaus, K., Agrawal, R.K. and Frank, J. (2002) EMBO J. 21, 3557–3567.