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

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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.

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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 [3]. 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 \(P_{\text{lac}}\) promoter and a \(LacI\)-\(O_{\text{miz}}\) 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 (\(tufA(Kir-R), tufB(B_{W})\)) [11], and DH5\(\alpha\). 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 \(\mu\)g/ml ampicillin, 25 \(\mu\)g/ml tetracycline, 30 \(\mu\)g/ml kanamycin or 50 \(\mu\)g/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’-GCTGAGATGCGTCTAAGGAAATATT-GAAGG-3’: sequence homology with the template is underlined) was used as upstream primer and SR-8 (5’-GTCGGATCCGCGCCCTTCTTTTTT-3’) as downstream primer.

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CAATTCCAAAC-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'-ATAGGATCCGCCC- GAAACTTTAGACAAAC-3') as downstream primer. The reaction mixture with 2 mM MgCl₂ 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, Gibeo Life Sciences, and MBI Fermentas. DNA fragments were isolated using Qiaex II Gel Extraction Kit (Qiagen). Plasmid preparations were done using Qagen and Jetprep plasmid preparation kits. PCR-amplified tufA sequences with or without the original stop codon were cloned into pTREC99A (Pharmacia Biotech), using flanking upstream EcoRI and downstream BamHI restriction sites, resulting in the plasmids pTRCtufA and pTRCtufACEX, respectively. The tetracycline-resistant variant of pTRCtufACEX is pTRCtufACEXTc, which confers tetracycline but not ampicillin resistance. To construct the latter plasmid, the unique FspI site within the bla gene was used to insert an EcoRI-StyI fragment of pBR322 encoding the tet gene with blunt ends filled in with Vent-polymerase.

In order to construct C'-terminal extensions to EF-Tu, a filled-in downstream BamHI site in pTRCtufACEX was used to join the tufA coding sequence to a blunt upstream end of Z domain multimer-coding fragments generated by FspI 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. HindIII sites at the downstream end of the Z multimer fragments were used to construct tuaZ-A2, tuaZ-A3, and tuaZ-A2 gene fusions in plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ2 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 2.5% (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 OD₅₇₀.

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 plKNG101-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 resistance gene of the plasmid pUC4K (Pharmacia) with flanking EcoRI sites, resulting in the strain SR92 (ΔtufB::kan). Strain AZ32 (ΔtufA::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 Inouye 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 pTRCtufACEXTc and plated on tetracycline/IPTG plates to select for the latter plasmid. The transformants were screened for ampicillin sensitivity to indicate the loss of pTRCtufACEXTc. Next, SR143 cells harboring the plasmid pTRCtufACEXTc were transformed with plasmids pTRCtufAZ2, pTRCtufAZ3, pTRCtufAZ4 or pTRCtufAZ5, expressing resistance to ampicillin. Transformants were selected on ampicillin/IPTG plates and screened for tetracycline sensitivity to indicate the loss of pTRCtufACEXTc.

3. Results

An E. coli strain was constructed in which both chromosomal genes encoding EF-Tu have been precisely replaced by antibiotic resistance cassettes (ΔtufB::kan; ΔtufA::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 pTREC99A (Pharmacia Biotech), resulting in the plasmid pTRCtufA. In this expres-
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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal genotype</th>
<th>Plasmid</th>
<th>Doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>tufA+, tufB+</td>
<td>pTRC99A</td>
<td>32</td>
</tr>
<tr>
<td>SR92</td>
<td>tufB: kan</td>
<td>pTRC99A</td>
<td>35</td>
</tr>
<tr>
<td>AZ32</td>
<td>tufA::cat</td>
<td>pTRC99A</td>
<td>44</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufA</td>
<td>59</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufACEX</td>
<td>72</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufA2Z2</td>
<td>109</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufA2Z3</td>
<td>109</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufA2Z4</td>
<td>147</td>
</tr>
<tr>
<td>SR143</td>
<td>tufA::cat, tufB: kan</td>
<td>pTRCtufA2Z5</td>
<td>100</td>
</tr>
</tbody>
</table>

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

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-O_110 system (Fig. 1).

EF-Tu (pTRCtufA and pTRCtufACEX), the chromosomal tuf gene was replaced with a kanamycin phosphotransferase cassette (kan) while mycin-resistant EF-Tu (A_r) while the construct pTRCtufACEX-Tc and the resulting strain was SR143 (tufA::cat, 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. The resulting strain was SR143 (tufA::cat, tufB::kan) with either plasmid pTRCtufA or pTRCtufACEX.

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 pTRCtufA2Z2, pTRCtufA2Z3, pTRCtufA2Z4 and pTRCtufA2Z5 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, kirromycin sensitivity was tested for plasmid-expressed EF-Tu and C-terminally tagged variants in strain UI232 (A, B).

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amp</th>
<th>Amp, IPTG</th>
<th>Amp, Kir</th>
<th>Amp, Kir, IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRC99A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufACEX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufAZ2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufAZ3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufAZ4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTRCtufAZ5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

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 strain AZ32 (tufA::cat). The resulting strain was SR143 (tufA::cat, tufB::kan) with either plasmid pTRCtufA or pTRCtufACEX.
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.

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\(^\beta\)Tet\(^\beta\) 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 tufA or tufB 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\(_n\) 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

[Image: 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 (U1232/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-Z2, EF-Tu-Z3, EF-Tu-Z4 and EF-Tu-Z5, 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-Z2 and EF-Tu-Z3 protein bands were homogeneous on SDS gels, indicating that these constitutions are stable. In the case of the two longest extensions, -Z4 and -Z5, 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 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/pTRCtufAZ2 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 characteristic EF-Tu band in the 43 kDa molecular weight range. Instead, they gave a strong band corresponding to an EF-Tu-Z2 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...
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 (ΔtufB::kan; ΔtufA::cat).

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References