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Vijgenboom, E.; Bosch, L.

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Transfer of plasmid-borne *tuf* mutations to the chromosome as a genetic tool for studying the functioning of EF-TuA and EF-TuB in the *E. coli* cell

E. VIJGENBOOM and L. BOSCH*

Department of Biochemistry, Leiden University, Wiassenaarseweg 64, 2333 AL Leiden, The Netherlands

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Summary – The elongation factor EF-Tu of *E. coli* is a multifunctional protein that lends itself extremely well to studies concerning structure-function relationships. It is encoded by two genes: *tufA* and *tufB*. Mutant species of EF-Tu have been obtained by various genetic manipulations, including site- and segment-directed mutagenesis of *tuf* genes on a vector. The presence of multiple *tuf* genes in the cell, both chromosomal and plasmid-borne, hampers the characterization of the mutant EF-Tu. We describe a procedure for transferring plasmid-borne *tuf* gene mutations to the chromosome. Any mutation engineered by genetic manipulation of *tuf* genes on a vector can be transferred both to the *tufA* and the *tufB* position on the chromosome. The procedure facilitated the functional characterization of some of our recently obtained *tuf* mutations. Of particular relevance is, that it enabled us for the first time to obtain a mutant *tufB* on the chromosome, encoding an EF-TuB resistant to kirromycin. It thus became possible to study the consequences for growth of *tufA* inactivation by insertion of bacteriophage Mu. The preliminary evidence obtained suggests that an EF-TuA, active in polypeptide synthesis, is essential for growth whereas such an EF-TuB is dispensable.

elongation factor EF-Tu / mutated *tuf* genes / homologous recombination

Résumé – Transfert à un chromosome de mutations *tuf* portées par un plasmide en tant qu'outil génétique pour l'étude du fonctionnement d'EF-TuA et d'EF-TuB dans la cellule d'*E. coli*. Le facteur d'élongation EF-Tu d'*E. coli* est une protéine multifonctionnelle qui se prête extrêmement bien aux études concernant les relations structure-fonction. Ce facteur est codé par deux gènes *tufA* et *tufB*. Des espèces mutantes de EF-Tu ont été obtenues par diverses manipulations génétiques, comprenant la mutagenèse de gènes *tuf* sur un vecteur, dirigée vers un site ou un segment. La présence de multiples gènes *tuf* dans la cellule, aussi bien chromosomiques que portés par un plasmide, gêne la caractérisation du mutant EF-Tu. Nous décrivons une procédure permettant de transférer au chromosome des mutations du gène *tuf* porté par un plasmide. Toute mutation obtenue par manipulation génétique des gènes *tuf* sur un vecteur peut être transférée à la position *tufA* et *tufB* sur le chromosome. Cette procédure a facilité la caractérisation fonctionnelle de certaines de nos mutations *tuf* récemment obtenue. Il est particulièrement pertinent de noter que cela nous a permis, pour la première fois, d'obtenir sur le chromosome un mutant *tufB*, codant pour un EF-TuB résistant à la kirromycine. Il devient ainsi possible d'étudier les conséquences sur la crois-

* To whom correspondence should be addressed.

sance de l'inactivation de *tufA* par l'insertion du bactériophage Mu. Les données préliminaires obtenues jusqu'ici suggèrent qu'un EF-TuA, actif dans la synthèse des polypeptides, est essentiel à la croissance alors qu'on peut se passer d'un tel EF-TuB.

facteur d'élongation EF-Tu / gène *tuf* muté / recombinaison homologue

Introduction

In *E. coli* the polypeptide chain elongation factor Tu (EF-Tu) is encoded by two unlinked genes, designated *tufA* and *tufB* [1]. The presence of these two almost identical *tuf* genes, separated by almost 660 kilobases on the chromosome, is highly intriguing. Their nucleotide sequences differ at 13 positions only and their gene products EF-TuA and EF-TuB are identical except for the C-terminal amino acid residue [2]. No functional differences have been reported for the two proteins [3, 4].

This *tuf* gene duplication is not restricted to *E. coli* but on the contrary is widespread among gram-negative bacteria. Nine enteric and four nonenteric gram-negative genera were found to carry two *tuf* gene copies [5]. In *E. coli* *tufA* is linked genetically to *fus* and *rpsL*, while *tufB* is linked to *rpoB* [6]. The distance between the duplicate *tuf* genes on the chromosome is much smaller in two of the nonenteric gram-negative bacteria: *Pseudomonas* and *Caulobacter*. Here the *rpsL* and *rpoB* markers are linked. In the cyanobacterium *Spirulina platensis*, considered as a gram-negative organism, the two *tuf* genes have been located on the same 6 kb restriction fragment [7].

One single *tuf* gene copy only could be identified in two gram-positive bacteria. In one of them, *Bacillus subtilis*, *rpsL* and *rpoB* are linked.

Although the *tuf* genes of the organisms mentioned above display structural homology, almost nothing is known about their protein products. The near identity of EF-TuA and EF-TuB in *E. coli* may or may not be found in the other organisms.

One way of studying the functioning of the two *tuf* gene products is the isolation of *E. coli* mutants altered in EF-Tu. The finding of an antibiotic with a target site on EF-Tu [8] has greatly facilitated the isolation of such mutants [9–11]. Parmeggiani and coworkers demonstrated that the antibiotic kirromycin binds to EF-Tu in a 1:1 molar ratio, thus preventing the release of EF-Tu from the ribosome and immobilizing the ribosome on the polysome [12, 13]. Resistance to kirromycin therefore requires the alteration of both *tuf* genes and sensitivity to the antibiotic dominates resistance. Previously we have isolated a kirromycin resistant mutant of *E. coli*

harbouring a resistant *tufA* gene and a recessive *tufB* gene [9, 11]. Since resistance to kirromycin provides a strong selection marker, various new mutant strains could be derived from the original mutant by mutating *tufB* but leaving *tufA* unaltered. This enabled us to inactivate *tufB* by either an insertion of the bacteriophage Mu or by an amber mutation. Such mutants, carrying a non-functioning *tufB*, were viable and showed no prominent effect on growth. We concluded that *tufA* is sufficient for growth [14].

Obviously it would be of great interest to construct *E. coli* strains harbouring a kirromycin resistant *tufB* and generate new mutants affected in *tufA*. Mutants of this type not only would provide us with new altered species of EF-TuA but they also might shed light on the question of whether or not *tufA* is essential for growth.

We have made a major step towards this goal by developing a procedure to transfer plasmid-borne *tuf* mutations to the chromosome *via* homologous recombination. This made it possible to obtain mutant strains carrying a kirromycin resistant *tufB*. The procedure is of general significance, however, and allows the introduction into the chromosome of any *tuf* mutation, engineered by genetic manipulations of plasmid-borne *tufA* or *tufB*. We describe a few examples and present preliminary evidence that *tufA*, in contrast to *tufB*, is essential for growth.

Materials and methods

Strains and genetic procedures

The *E. coli* strains used in this study are listed in Table I. Transductions were performed with P1vir according to Miller [17]. The *polA*_{ts} allele was cotransduced with *metE* from strain KA414 to LBE2016. The transductants were selected on minimal medium without vitamin B12 (*metE* encodes for this vitamin) and the presence of the *polA*_{ts} mutation was demonstrated by sensitivity to UV light and 3 mM methylmethane sulfonate at 42°C. One of the transductants, EV1, was used for further constructions. Strain EV11 was constructed by transduction of the wild type *tufB* gene from LBE2041 to EV1. After selection for rifampicin (20 µg/ml) resistance (cotransduction of *tufB* and *rpoB* is about 80%), colonies were

Table I. *Escherichia coli* K12 strains used.

Strain	EF-Tu mutations	Genotype	Reference
KA 414		<i>thy, rho, lac, rpsL, polA12^a, su⁺</i>	^b
LBE 2012	A(ala 375→thr), B(gly 222→asp)	<i>xyl, tufA, tufB</i>	14
LBE 2016	A(ala 375→thr), B(gly 222→asp)	<i>xyl, metE, tufA, tufB</i>	14
LBE 2020		<i>rpoB, tufB</i>	14
LBE 2040		<i>fus</i>	14
LBE 2041		<i>rpoB</i>	14
PM 814	A(ala 375→thr)	<i>rpsL, tufA</i>	15
PM 816	A(ala 375→thr)	<i>fus, tufA</i>	15
PM 1455	A(ala 375→thr)	<i>recA, rpoB, tufA, tufB::(Mu)</i>	15
EV 4		<i>Δ(pro lac), rpoB, tufB::(Mu)</i>	16
EV 1	A(ala 375→thr), B(gly 222→asp)	<i>xyl, polA12, tufA, tufB</i>	This paper
EV 11	A(ala 375→thr)	<i>xyl, polA12, rpoB, tufA</i>	This paper
EV 13	A(ala 375→thr), B(ala 375→thr)	<i>xyl, polA12, rpoB, tufA, tufB</i>	This paper
EV 16		<i>xyl, polA12, rpoB, fus, tufB</i>	This paper
EV 100	A(ala 375→thr), B(ala 375→thr)	<i>rpoB, fus, tufA, tufB</i>	This paper
EV 102		<i>rpoB, fus, tufB</i>	This paper
EV 110	A ^c , B(gly 222→asp)	<i>rpoB, fus, tufA, tufB</i>	This paper
EV 113	A ^c	<i>Δ(pro lac), rpoB, fus, tufA, tufB::(Mu)</i>	This paper

^a *polA12* encodes the temperature sensitive mutant of DNA polymerase I.

^b Obtained from the collection of the Department of Molecular Genetics, State University, Leiden, The Netherlands.

^c The amino acid replacement in *tufA* is unknown, this mutant is referred to as *tufA*^{*}.

screened for kirromycin sensitivity. Transduction of the wild type *tufA* gene from strain LBE2040 to EV13 and selection for fusidic acid (500 µg/ml) resistance (cotransduction of *tufA* and *fus* is about 90%), resulted in strain EV16. The isolation of the other EV-strains is described in the Results.

Plasmid construction

The plasmid pVE2 was constructed by ligating the 4.0 Kb EcoRI fragment of pGp82^R (identical to pGp82 [15] except for the *tufA* (ala 375→thr) mutation present on pGp82^R) in the EcoRI site of pBR329 [18]. The *tufA* (ala 375→thr) on pVE2 is in the same orientation as the Tc^R gene. The plasmid pVE6 consists of a PstI fragment harbouring the *tufB* (gly 222→asp) gene cloned in the PstI site of pBR329. The *tufB* (gly 222→asp) gene is in the same orientation as the Cm^R gene of pBR329. The plasmid pVE8 is a derivative of pBR329 with the 4.0 Kd EcoRI fragment of pGp82A* (compare results) in the EcoRI site of pBR329. The orientation of *tufA** is opposite to that of *tufA* (ala 375→thr) in pVE2. The plasmid pVE24 is a pBR322 [19] derivative harbouring the *tufB* (ala 375→lys) gene and was constructed as follows. The BamHI-NruI fragment of pTuB11.1 [20] harbouring *tufB* was ligated in BamHI-HincII digested M13mp18 dsDNA. After oligonucleotide-directed mutagenesis, an EcoRI-PstI fragment, cut at the polylinker sites and containing the *tufB* gene, was cloned in EcoRI, PstI digested pBR322. Plasmid- and fragment- isolations, digestions

with restriction endonucleases and transformations were done according to standard methods.

Media

LB medium contained per liter: 10 g tryptone, 8 g NaCl, 5 g yeast extract, pH 7.0. Fusidic acid resistant transductants were isolated on minimal medium [21] supplemented with 0.5% of glucose, 1% Cas-amino acids, 1 mM EDTA and 500 µg/ml fusidic acid. Fusidic acid was generously provided by Leo Pharmaceutical Products Ltd. Rifampicin resistant transductants were isolated on the same minimal medium with 20 µg/ml of rifampicin.

Transfer of plasmid-borne *tuf* mutations to the chromosome

The strains EV11 and EV16 were transformed with derivatives of pBR322 or pBR329, and selection was for resistance to 10 µg/ml of tetracycline on LB medium at 28°C. One transformant was grown overnight in LB medium with 10 µg/ml of tetracycline at 28°C. The morning after the culture was diluted 1:100 in LB + 10 µg/ml of tetracycline and grown at 28°C. At OD₆₀₀ = 0.25 the temperature was shifted to 42°C and incubation was continued overnight. A 1:100 diluted culture was grown in the presence of 10 µg/ml of tetracycline for two generations, cells were spun down and washed twice in fresh medium of 42°C. Resuspended cells were grown for at least ten generations in LB without tetracycline at 42°C. Enrichment of tetracycline sensitive cells was achieved

by three successive cycloserine treatments according to Bolivar *et al.* [19]. Kirromycin resistant colonies were isolated on LB plates with 50 $\mu\text{g}/\text{ml}$ kirromycin and 1.5 mM EDTA. These colonies were characterized by P1 mediated transductions and isoelectric focusing of 'S30' extracts.

Other procedures

Rocket immunoelectrophoresis was performed with crude cell lysates according to van der Meide *et al.* [22]. Isoelectric focusing of 'S30' extracts was according to O'Farrell *et al.* [23]. Oligonucleotide directed mutagenesis was carried out with M13 ssDNA isolated according to Kunkel [24]. After hybridization of the oligonucleotide to the ssDNA, the gap was filled with dNTP's, Klenow and T4 ligase in 20 mM Hepes pH 7.8, 5 mM DTT, 10 mM MgCl_2 , 0.2 mM ATP and 0.125 mM of each of the four NTP's. Incubation was for 90 min at 14°C and 15 min at 37°C. Half of the reaction mixture was used to transfect JM101 or JM105 [25]. Screening of the correct mutant was done by sequencing with the dideoxy method according to Sanger *et al.* [26].

Results

Tuf mutations to be transferred

In our earlier investigations we studied two mutations of *tufA* that confer resistance to kirromycin as a result of the replacement of ala-375 by threonine [27] and valine [28], respectively. Crystallographic data reported by Rubin *et al.* [29] indicated that ala-375 is located in a region profoundly involved in the allosteric control of the EF-Tu molecule. A preliminary interpretation of the electron densities located residue 375 in the cleft formed between the N-terminal and the C-terminal domain of EF-Tu. We have assumed, therefore, that substitution of ala-375 by threonine or valine alters the position of the N-terminal and the C-terminal domain relative to each other. For this reason it seemed of interest to substitute ala-375 by lysine and to study the phenotypical consequences. Recently, we have achieved such a substitution by oligonucleotide-directed mutagenesis of *tufB* (our unpublished results). So far the properties of EF-TuB (ala 375 \rightarrow lys) have not been investigated in detail. We refer to the various mutations as *tufA* (ala 375 \rightarrow thr), *tufA* (ala 375 \rightarrow val) and *tufB* (ala 375 \rightarrow lys).

Another mutation affecting *tufB* was originally obtained by isolating the mutant strain LBE 2012. The *tufB* mutation substitutes gly-222 by aspartic acid and shifts the isoelectric point of the protein 0.1 pH unit to the acidic side. The mutation has been cloned on the plasmid pVE6. Strain LBE 2012

harbours both *tufA* (ala 375 \rightarrow thr) and *TufB* (gly 222 \rightarrow asp) on the chromosome. *TufB* (gly 222 \rightarrow asp) is recessive to *tufA* (ala 375 \rightarrow thr) and the strain LBE 2012 is therefore resistant to kirromycin [9, 11].

Finally a spontaneous mutant arose that harbours a mutation on a plasmid-borne *tufA*. The strain is kirromycin resistant and carries a kirromycin resistant *tufA* on the chromosome while the chromosomal *tufB* has been inactivated by insertion of bacteriophage Mu. The mutation has not been characterized in detail and is referred to as *tufA*^{*} (located on pGp82A^{*}). Isoelectric focusing revealed that the isoelectric point of EF-TuA^{*} is shifted 0.05 pH unit to the alkaline side (compare for instance Fig. 2).

General outline of the procedure for transferring plasmid-borne tuf mutations to the chromosome

In the following we describe the transfer of a kirromycin resistant mutation of a plasmid-borne *tufA* gene to a wild type chromosomal *tufB*. The procedure allows in principle, however, to transfer all kinds of plasmid-borne *tuf* mutations to either the *tufA* or the *tufB* position on the chromosome. The procedure is based on a method described by Gutteresen and Koshland [30] adapted to the present aim (see also Greener and Hill [36]). It makes use of a strain defective in DNA polymerase I in which CoIEI-derived recombinant plasmids are unable to replicate extrachromosomally. A plasmid (pEV2) carrying a tetracycline resistance marker and a kirromycin resistant *tufA* was therefore introduced at the permissive temperature into a polA_{ts} strain (EV11) carrying a kirromycin resistant *tufA* on the chromosome. At the non-permissive temperature cells can only survive in the presence of tetracycline upon integration of the plasmid into the chromosome. Integration via homologous recombination occurs thanks to the large homology between *tufA* and *tufB*, and leads to a non tandem duplication of *tuf* sequences separated by vector sequences (compare Fig. 1). Elimination of the wild type *tufB* sequence is then achieved by a second recombination event in the presence of tetracycline and cycloserine at the non-permissive temperature. This procedure selects for non growing tetracycline-sensitive strains that have lost all vector sequences and carry either the original chromosomal *tufB* or the mutant *tufB* gene. Screening of kirromycin resistant mutants yields strains that are completely isogenic with the parental strain except for the *tufB* gene. Use of a polA_{ts} strain instead of a polA^- (as originally described by Gutteresen and Koshland

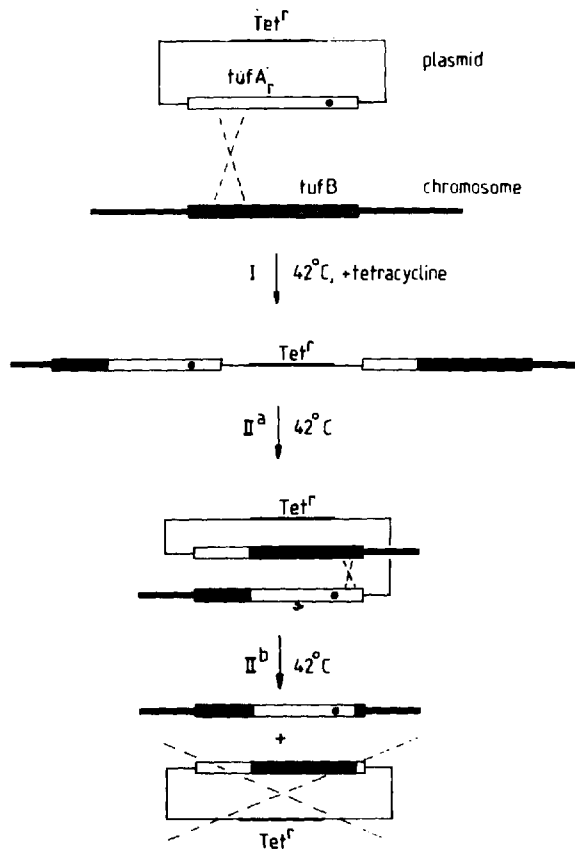


Fig. 1. Transfer of a mutation (black spot) from a plasmid-borne to a chromosomal *tuf* gene. Step I represents the integration of the plasmid into the chromosome by a homologous recombination event, symbolized by the dashed lines. The result is a duplication of the *tuf* sequences on the chromosome separated by the vector sequences. Step II^a and II^b represent the segregation of the plasmid from the chromosome by a second homologous recombination event. If this recombination takes place as indicated in the figure the mutation will be preserved on the chromosome. If it occurs at the other side of the black spot, no transfer to the chromosome takes place and the mutation remains on the plasmid. The plasmid that is resolved from the chromosome after step II^b will be lost at the non-permissive temperature. As illustrated the chromosomal *tuf* gene is composed of three portions, two derived from the original chromosomal *tuf* gene (black) and one from the plasmid-borne *tuf* gene (white). Since there is no homology in the sequences flanking the *tufA* and *tufB* gene, the N⁻ and C-terminal codons will always remain on their original *tuf* gene. An EF-TuB producing gene therefore cannot be changed into an EF-TuA producing gene by this procedure.

[30]) permits the accumulation in the cell of a sufficient number of plasmid copies at the permissive temperature, whereafter homologous recombination can be induced by temperature elevation to the

non-permissive level. Because of the high mutation frequency of *polA_{ts}* strains, all mutant *tuf* genes thus acquired have to be transferred to *polA⁺* strains by P1-mediated transduction as soon as the *polA_{ts}* allele is no longer needed. The procedure has been applied successfully to construct strains EV13 and EV100.

Insertion of bacteriophage Mu into chromosomal *tufA*

As pointed out in the Introduction, insertion of bacteriophage Mu into *tufB* had a minor effect on growth only. Such an insertion was achieved by infecting cells, carrying a kirromycin resistant *tufA* and a wild type *tufB*, with Mu and selecting for kirromycin resistance. Resistant cells were screened for Mu lysogeny.

The procedure outlined in the previous paragraph enabled the construction of cells harbouring a wild type *tufA* and a kirromycin resistant *tufB* and provided the possibility to study the consequences of *tufA* inactivation. With this aim the kirromycin resistant *tufA* (*ala 375*→*thr*) of strain EV100 (Table I) was first replaced by its wild type counterpart by a P1-mediated transduction and the resulting strain EV102 was then infected with Mu. None of the colonies selected for kirromycin resistance were found to be Mu resistant. In contrast, as found before, Mu infection of control strains (PM816), harbouring a *tufA* (*ala 375*→*thr*) and a wild type *tufB*, did yield significant numbers of colonies resistant to both kirromycin and Mu. These data indicate that inactivation of *tufA* is lethal to the cell, whereas inactivation of *tufB* does not lead to loss of viability.

Transfer of the *tufB* mutation: *gly 222*→*asp* from the plasmid to the chromosomal *tuf* genes

Homologous recombination between mutant *tufB* (*gly 222*→*asp*) located on the plasmid pEV6 and wild type chromosomal *tufB* in cells of strain EV11, harbouring a mutant chromosomal *tufA* (*ala 375*→*thr*) yielded the expected results. Recombinants resistant to kirromycin could be selected and 'S30' extracts from the recombinant cells yielded two EF-Tu electroisomers upon isoelectric focusing, differing 0.1 pH unit in isoelectric point (compare Fig. 2).

Noteworthy, however, is that transfer of the *gly 222*→*asp* mutation to the chromosomal *tufA* of strain EV16 did not yield viable cells. These data indicate that cells with the chromosomal *tuf* gene combination:

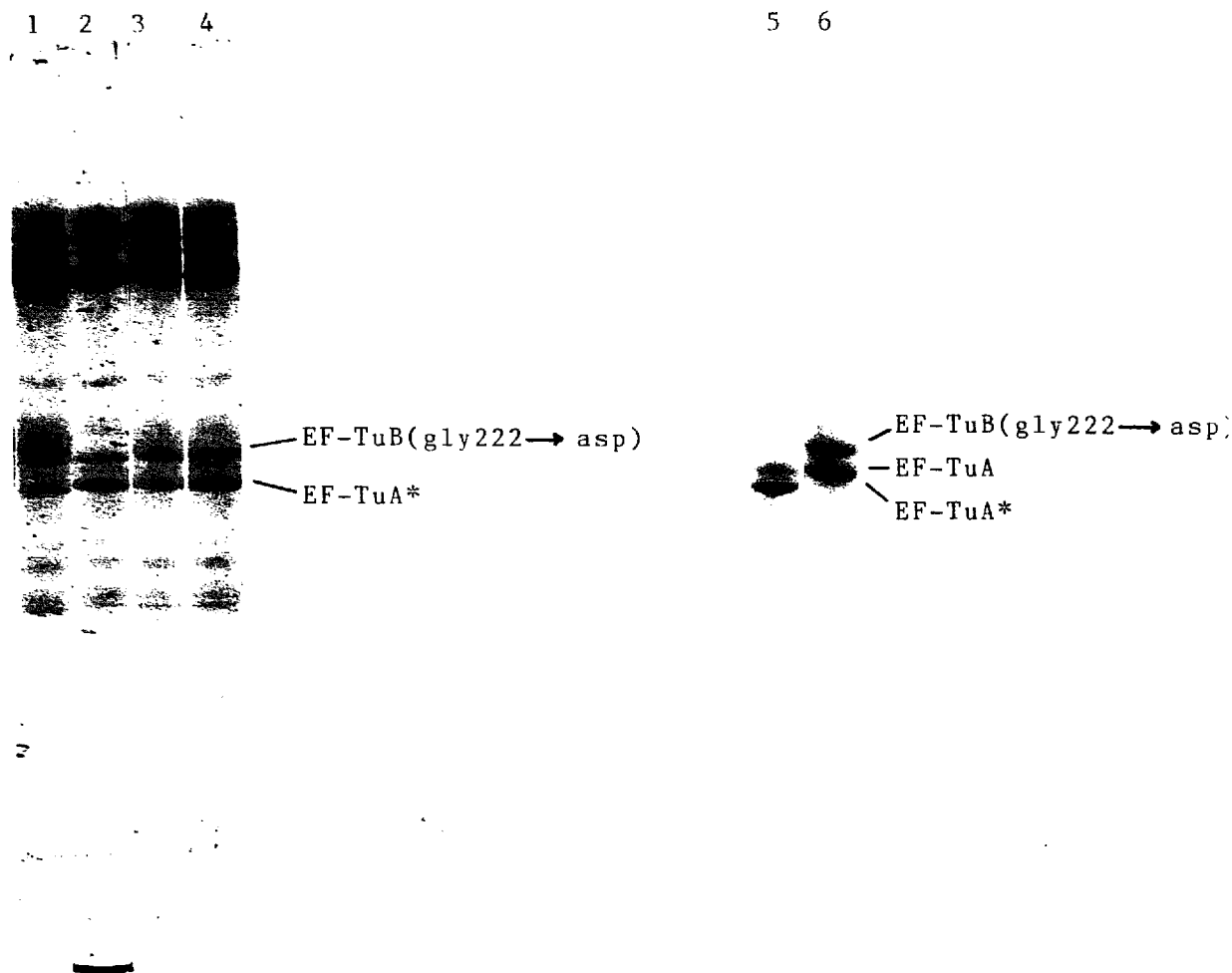


Fig. 2. Isoelectric focusing 'S30' extracts (gels 1-4) and EF-Tu purified by means of affinity chromatography [31] (gels 5 and 6). Gels 1-4 contain 100 μ g protein of four independent isolates of strain EV110. Gels 5 and 6 contain 10 μ g of purified EF-TuA + EF-TuA* and EF-TuB (gly 222→asp) + EF-TuA* respectively.

TufA (ala 375→thr), *tufB* (gly 222→asp) are viable whereas cells with the combination: *tufA* (gly 222→asp), *tufB* (ala 375→thr) are not (see further the Discussion).

Transfer of the tufA mutation from the plasmid to chromosomal tufA*

Transfer of the *tufA** mutation from the plasmid pVE8 to wild type chromosomal *tufA* of strain EV16, harbouring a kirromycin resistant chromosomal *tufB* (ala 375→thr) aided in the functional characterization of EF-TuA*. After the recombination event kirromycin resistant colonies could be selected, indicating that the EF-TuA*, encoded

by the transferred *tufA**, is either kirromycin resistant or unable to sustain polypeptide synthesis.

The *tufA** gene, now located on the chromosome, was then cotransduced with Fus^R (compare Table I) to the strain LBE2020, harbouring wild type *tufA* and mutant *tufB* (gly 222→asp). Cotransductants, resistant to both fusidic acid and kirromycin, could be selected, demonstrating that the protein product of *tufA** is resistant to kirromycin. The presence of this product EF-TuA* in cell-free 'S30' extracts could be demonstrated by isoelectric focusing. As illustrated in Figs. 2 and 3 the isoelectric point of EF-TuA* is shifted 0.05 pH unit to the alkaline side when compared to that of wild type EF-Tu and 0.15 pH unit when com-

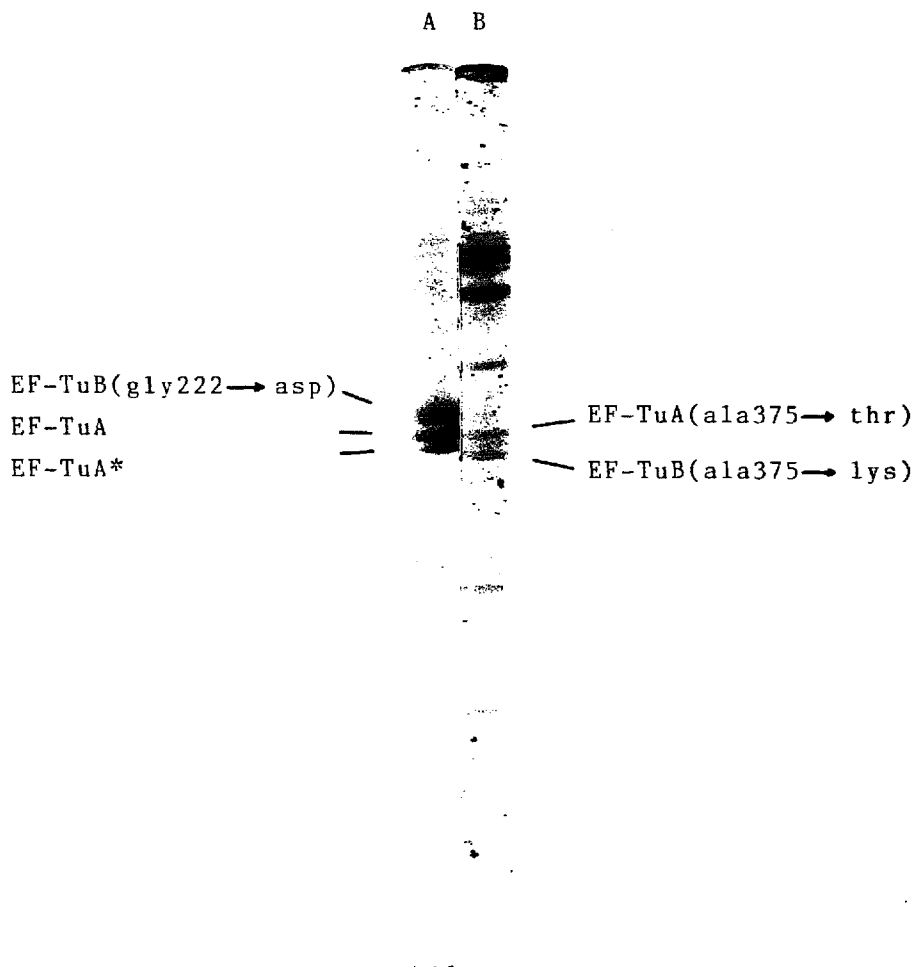


Fig. 3. Isoelectric focusing of purified wild type and mutant EF-Tu (lane A) and an 'S30 extract' (100 μ g protein) from strain PM1455 harbouring the overproducing derivative of pVE24 (lane B). These gels illustrate the differences in isoelectric point of the various EF-Tu species.

pared to the isoelectric point of EF-Tu (gly 222→asp). The finding that *tufA** is expressed in cells harbouring the mutant *tufB* (gly 222→asp) leading to a kirromycin resistant phenotype, demonstrates that the mutant EF-TuA* species is active in protein synthesis and displays a reduced affinity for kirromycin. This has been confirmed by transduction of *tufA** to a strain (EV4), carrying a *tufB* inactivated by insertion of bacteriophage Mu. The resulting strain (EV113) is kirromycin resistant and produced EF-TuA* as the sole *tuf* gene product. This enables the isolation of this product in a homogeneous state and detailed characterization of its functions.

Transfer of the tufB mutations: ala 375→lys from plasmid to the chromosomal tufA gene

The *tufB* mutation: ala 375→lys was constructed by oligonucleotide-directed mutagenesis and cloned in pBR322. The mutant *tufB* gene on the resulting plasmid pEV24 is under the control of a weak promoter. When introduced into the kirromycin resistant strain PM1455 ($A_R B::Mu$) it was moderately expressed as was demonstrated by rocket immunoelectrophoresis (Fig. 4). The molar ratio of EF-TuA (ala 375→thr) and EF-TuB (ala 375→lys) ratio was determined and found to be 7:2 (Table II). The presence of EF-TuB (ala 375→lys)

Table II. Relative concentrations of EF-TuA, EF-TuB and EF-Ts in strain PM 1455 and some of its transformants.

Strain	Total EF-Tu/EF-Ts	EF-TuA (ala 375→thr) / EF-TuB (ala 375→lys)
PM 1455	5.3	—
PM 1455 pVE24	6.8	3.5
PM 1455 pVE24.11*	15.0	0.55

See also the legend to Fig. 4.

* pVE24.11 is the plasmid with a strong promoter cloned upstream of the mutant *tufB* gene.

did not alter the phenotype of strain PM 1455 ($A_R B::Mu$) indicating that it does not cause immobilisation of the ribosomes on the messenger. Elevation of the cellular concentration of EF-TuB (ala 375→lys) (molar ratio EF-TuA (ala 375→thr): EF-TuB (ala 375→lys) of 5:10) by putting the mutant *tufB* gene on the plasmid behind a different promoter, resulted in growth inhibition. These results (to be published) suggest that EF-TuB (ala 375→lys) is toxic for the cell. EF-TuB (ala 375→lys) has an isoelectric point that is shifted 0.1 pH unit to the alkaline side which is demonstrated by isoelectric focusing (Fig. 3).

Transfer of the *tufB* mutation from pEV24 to the chromosomal *tufA* was attempted in strain EV16. The number of colonies found to be resistant to kirromycin after cycloserine enrichment did not exceed that of a control experiment with a wild type *tufB* gene on the plasmid. Isoelectric focusing of 'S30' extracts of these kirromycin resistant strains did not reveal the presence of an electroisomer corresponding to EF-TuA (ala 375→lys). These results suggest that transfer of the ala 375→lys mutation to chromosomal *tufA* is incompatible with growth.

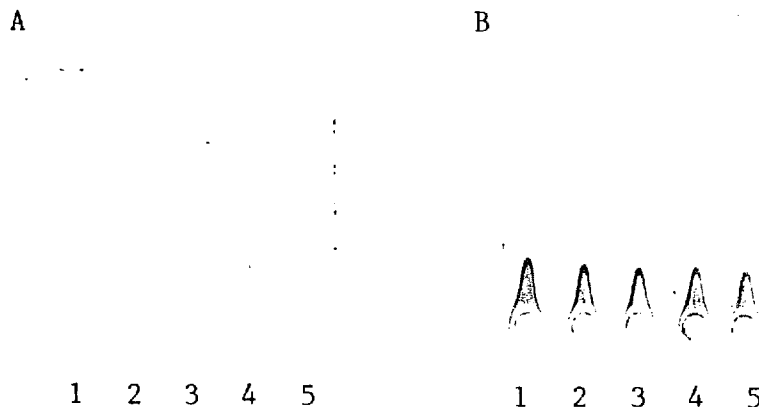


Fig. 4. Rocket immunoelectrophoresis of 'crude extracts' of strain PM1455 (rocket 1), PM1455 pVE24 (rocket 2 and 4) and PM1455 harbouring the EF-TuB (ala 375→lys) overproducing derivative of pVE24 (rocket 3 and 5). The gel in panel A contains polyclonal antibodies raised against EF-Tu and the gel in panel B polyclonal antibodies raised against EF-Ts. The amount of 'crude extract' applied to the wells was seven times higher in panel B than in panel A. The concentration of EF-Tu and EF-Ts can be determined as described by van der Meide *et al.* [22]. The intracellular concentration of EF-Ts is used as an internal standard (see also Table II).

Discussion

The multifunctional character of EF-Tu, its moderate size and the availability of cloned *tuf* genes makes this protein an ideal object for studies of structure-function relationships. A complicating factor, however, is the presence of two *tuf* genes. Characterization of mutant EF-Tu species thus requires the separation of proteins that are structurally almost identical [2, 32, 33]. We have overcome this difficulty in some cases, for instance by inactivating *tufB*. Cells thus treated produce EF-TuA as the sole *tuf* gene product. So far it has not been possible, however, to inactivate *tufA* without loss of viability (see also below). Moreover, site-directed mutagenesis of cloned *tuf* genes inevitably will require the introduction of additional *tuf* genes into the cell. Reduction of the number of *tuf* genes by transfer of plasmid-borne genes to the chromosome may thus contribute significantly to the isolation of single-gene products in a homogenous state. Such a transfer may also be a requisite in studies dealing with the regulation of expression of normal and mutant *tuf* genes.

The procedure described in this paper for transferring plasmid-borne *tuf* gene mutations to chromosomal *tuf* genes suits this purpose well. Transfer to both the *tufA* and the *tufB* positions on the chromosome is possible. Furthermore, any mutation engineered by genetic manipulation of *tuf* genes on a vector can be transferred. In fact transfer can aid in the functional characterization of the mutation, as has been demonstrated here for *tufA*^{*} and *tufB* (ala 375→lys). After transfer of *tufA*^{*} to the chromosome the mutant *tuf* gene appeared to encode an EF-Tu species active in protein synthesis and with a lowered affinity for kirromycin. Over-expression of *tufB* (ala 375→lys) led to inhibition of cell growth, suggesting that the mutant gene product is toxic. Accordingly, transfer of *tufB* (ala

375→lys) to *tufA* on the chromosome did not yield viable cells.

A particular merit of the procedure is that it enabled us for the first time to obtain a mutant *tufB* gene on the chromosome, encoding an EF-TuB resistant to kirromycin. It thus becomes possible to answer the question of whether or not *tufA* is essential for growth. Cells harbouring this mutant *tufB* (ala 375→thr) gene and a wild type *tufA* were infected with the bacteriophage Mu. Since insertion of Mu into *tufA* should alter the phenotype, it was attempted to select colonies that displayed resistance to both kirromycin and Mu. No such colonies could be obtained. This result is in clear contrast to that of previous experiments [14] in which cells harbouring a kirromycin resistant *tufA* displayed the expected phenotype after insertion of Mu into wild type *tufB*. The preliminary conclusion is that *tufA* is essential and *tufB* by itself is unable to sustain growth.

If this can be substantiated by further experiments it means that EF-TuA and EF-TuB differ in function due to a structural difference of one single amino acid located at the C-terminus.

Interestingly, another observation of the present study also points to the importance of this C-terminal difference. Attempts to transfer the mutation: gly 222→asp to the *tufA* gene on the chromosome failed whereas such a transfer to the chromosomal *tufB* gene yielded viable cells. The implication may be recognized more readily by comparing that EF-Tu species of the viable and non-viable cells listed in Table III.

Loss of viability upon inactivation of chromosomal *tufA* would mean that EF-TuB by itself is unable to sustain polypeptide synthesis. That it does participate in protein synthesis in cells harbouring a kirromycin resistant *tufA* can be concluded from the fact that these cells are sensitive to the antibiotic. Apparently, EF-TuB is active

Table III. Viable and non-viable *tuf* gene combinations.

Genotype	Amino acid differences of:		Viability
	EF-TuA	EF-TuB	
<i>tufA</i> (ala 375→thr), <i>tufB</i> (gly 222→asp), <i>tufA</i> (gly 222→asp), <i>tufB</i> (ala 375→thr)	gly 393, thr 375	ser 393, asp 222	+
	gly 393, asp 222	ser 393, thr 375	-

when EF-TuA is also present. Previous studies by Miller *et al.* [4] indicated that there is little or no difference in the capacities of the products of *tufA* and *tufB* genes to take part in the reactions of peptide chain elongation. The activity of each separate gene product was investigated, however, in UV-irradiated *E. coli* cells infected by transducing phages bearing the separate genes. Apparently both gene products were present under these circumstances. Gausing described a *tufA* defective strain of *E. coli*, the growth rate of which was significantly decreased [34, 35]. The strain had a residual *tufA* expression of 10%. So far no viable strains have been reported with a fully inactivated *tufA*.

Obviously the preliminary data of the present investigations raise many questions concerning the functioning of EF-TuA and EF-TuB. The genetic tools described here have paved the way, however, for additional studies dealing with these important questions. These studies are now in progress and the results will be published elsewhere.

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