A kirromycin-resistant EF-Tu species reverses streptomycin dependence of *Escherichia coli* strains mutated in ribosomal protein S12

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Streptomycin dependence can be caused by mutations in ribosomal protein S12. Mutations suppressing such streptomycin dependence have been found in ribosomal proteins S4 and S5, and in 16S rRNA. Here a new suppressor mutation localized in elongation factor Tu (EF-Tu) is described, consistent with recent models of ribosome-EF-Tu-tRNA interaction at the decoding centre. The EF-Tu mutation was obtained by genetic selection for streptomycin independence; it was identified as Ala375 → Thr, previously described as EF-TuA_R and known to confer a kirromycin-resistant, error-prone phenotype. Also, other streptomycin-dependent (Sm^D) S12 mutations could be complemented by this mutation. The streptomycin-independent (Sm^I) strain grows more slowly than the wild-type (wt), suggesting that not all the defects of the S12 mutation can be complemented by EF-Tu[A375T]. Moreover, this strain is more susceptible than wt to reduction in the cellular EF-Tu concentration, and disruption of *tufB* led to considerable growth-rate impairment. Expression of EF-Tu from *tufB*, not only of wt EF-Tu and EF-Tu[A375T] but, remarkably, also of EF-Tu[G222D], known as EF-TuB₀ and defective in protein synthesis, equally contributed to cell growth. *In vitro* analysis revealed a decreased translational activity of wt EF-Tu with Sm^D ribosomes as compared to EF-Tu[A375T], while EF-Tu[G222D] showed no activity at all, just as with wt ribosomes. Possible mechanisms are discussed for the improved growth rate observed in such Sm^I strains when they include wt EF-Tu or EF-Tu[G222D].

Keywords: elongation factor Tu, error-prone mutations, *rpsL*, translational accuracy

INTRODUCTION

One of the key components in protein biosynthesis in *Escherichia coli* is the elongation factor Tu (EF-Tu), encoded by two almost identical genes, *tufA* and *tufB*. It is a member of the GTPase superfamily and complexes aminoacyl-tRNA (aa-tRNA) in the GTP-bound state. The ternary complex formed binds to a programmed ribosomal A-site and after correct codon-anticodon recognition GTP is hydrolysed, followed by the release

of EF-Tu · GDP from the ribosome (Miller & Weissbach, 1977). The EF-Tu binding site at the ribosomal A-site is located on both subunits. On the 30S subunit it is in the vicinity of ribosomal proteins S4, S5 and S12, and the 530 loop of 16S rRNA. On the 50S subunit it is close to ribosomal proteins L10 and L11, the L7/L12 stalk, the α -sarcin stem-loop structure and the thiostrepton binding structure of 23S rRNA (Saarma *et al.*, 1997; Stark *et al.*, 1997 and references therein). The interplay between EF-Tu and these ribosomal components controls translational accuracy during protein synthesis.

The antibiotic streptomycin interferes with the translation process by inducing misreading. Footprinting studies have shown that streptomycin binds to 16S rRNA at the A-site region of the decoding centre (Spickler *et al.*, 1997). Resistance to streptomycin can be obtained by mutations in 16S rRNA (Powers & Noller,

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Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; EF-TuA_S or EF-TuB_S, wild-type EF-Tu from *tufA* or *tufB*, respectively; EF-TuA_R or EF-TuB_R, EF-Tu[A375T] from *tufA* or *tufB*, respectively; EF-TuB₀, EF-Tu[G222D] from *tufB*; Sm^D, Sm^I, Sm^R or Sm^S, streptomycin-dependent, -independent, -resistant or -sensitive; Tc^R, tetracycline-resistant; wt, wild-type.

1991 and references therein), but also by mutations in ribosomal protein S12 (Ozaki *et al.*, 1969). Streptomycin-resistant (Sm^R) mutations in S12 (encoded by *rpsL*) can lead to hyperaccuracy and decreased activity in protein synthesis, and even to dependence on the error-inducer streptomycin (Birge & Kurland, 1969). These streptomycin-dependent (Sm^D) mutations are clustered in two regions, one spanning codons 40–43, the other 87–93 (Timms *et al.*, 1992). The Sm^D phenotype is not caused by hyperaccuracy alone since some of the Sm^R mutants that do not require streptomycin are more error-restrictive than Sm^D mutants (Ruusala *et al.*, 1984; Andersson *et al.*, 1986). Quench-flow analysis has shown that Sm^D ribosomes, in addition to the defects they have in common with Sm^R ribosomes, are perturbed in a step between peptidyl transfer and translocation or after translocation and before binding a new ternary complex (Bilgin *et al.*, 1992).

Streptomycin dependence can also be generated in other ways, for example by (i) combining a hyperaccurate Sm^R S12 mutation with the 1491C mutation in 16S rRNA (causing a paromomycin-resistant and slow-growth phenotype) (O'Connor *et al.*, 1991), (ii) combining a hyperaccurate Sm^R S12 mutation with a *miaA* mutation (causing a defect in a tRNA modification enzyme which reduces the efficiency of UAG/UGA read-through) (Petrullo *et al.*, 1983), or (iii) combining a pseudo-dependent S12 mutant with the error-restrictive 2661C mutation in 23S rRNA (Bilgin & Ehrenberg, 1994).

Streptomycin dependence of S12 mutants can be suppressed by *ram* (ribosomal ambiguity) mutations in ribosomal proteins S4 and S5, and in 16S rRNA (Birge & Kurland, 1970; Deuser *et al.*, 1970; Allen & Noller, 1991; Lodmell & Dahlberg, 1997). In the absence of the Sm^D S12 mutation these *ram* mutations cause a phenotype of high-level misreading.

Translational accuracy can also be affected by mutant EF-Tus, such as kirromycin-resistant EF-Tu A_R (A375T), EF-Tu A^* (later identified as Q124K) and the null mutant EF-Tu B_0 (G222D), which were originally found in kirromycin-resistant strains (Duisterwinkel *et al.*, 1981, 1984; Zeef & Bosch, 1993) and promote frameshifting (Hughes *et al.*, 1987; Vijgenboom & Bosch, 1989). The subscripts in A_R and B_0 refer to their effect on the kirromycin phenotype, i.e. sensitive for wild-type (wt) cells with EF-Tu A_S and EF-Tu B_S (for further explanation, see van der Meide & Vijgenboom, 1981). When EF-Tu A_R or EF-Tu A^* is combined with EF-Tu B_0 , the frequency of frameshifting exceeds the sum of the separate contributions (Vijgenboom & Bosch, 1989). Such a synergistic effect between EF-Tu A_R and EF-Tu B_0 has also been observed for nonsense suppression (Vijgenboom *et al.*, 1985). The increase in frameshifting and nonsense suppression caused by EF-Tu B_0 is striking because this mutant species by itself is not active in protein synthesis (Swart *et al.*, 1987; Talens *et al.*, 1996).

Since EF-Tu, together with other ribosomal components, is involved in maintaining translational ac-

curacy and binds in the vicinity of S12 at the ribosome, we tried to find mutations in EF-Tu that were able to suppress streptomycin dependence of S12 mutants. We found that the above-mentioned error-prone EF-Tu A_R mutation can also cause suppression of streptomycin dependence. This mutation has been reported to complement growth defects of hyperaccurate Sm^R S12 mutants (Tapio & Isaksson, 1988; Tubulekas & Hughes, 1993).

METHODS

Strains, media and genetic procedures. The *E. coli* strains used in this study are listed in Table 1. The chromosomal *tuf* genes (*tufA* and *tufB*) of Sm^D strain UD666 (harbouring the *rpsL666* allele) were subjected to random mutagenesis by M13-mediated allelic replacement (Zeef & Bosch, 1993). A tetracycline-resistant (Tc^R) gene linked to the *tuf* gene on the M13 phage (M13mp9Zam20C) enables selection for integration of the phage into the host genome by homologous recombination with one of the two *tuf* genes. The following deviations from the standard protocol were made. M13 lysogens were formed in medium containing 50 μg streptomycin ml^{-1} , followed by incubation at 37 °C for 2 h after removal of the unadsorbed phages. Selection for streptomycin-independent (Sm^I) strains was on LC plates (Zeef & Bosch, 1993) containing 50 μg tetracycline ml^{-1} and lacking streptomycin. To identify the *tuf* mutation in the Sm^I strain LZ18L, M13 retrieval was performed. Since retrieval of the mutation would make the strain Sm^D again, strain LZ18L was first transformed with plasmid pNO1523 (encoding wt S12) (Dean, 1981), which improves the growth rate of this strain, but more importantly makes an Sm^D strain sensitive to streptomycin. The mutation was identified by sequencing the retrieved M13 using T7 DNA polymerase (Pharmacia), [α - ^{32}P]dCTP (ICN) and *tuf* primers (Zeef & Bosch, 1993).

The transducing bacteriophage P1 was used for all transductional crosses (Miller, 1972). Transformation of strain LZ18L with plasmid pNO1523 also facilitated the preparation of a P1 lysate from this strain. This P1 lysate was used to determine which *tuf* gene was mutated in LZ18L by infecting the fusidic-acid-resistant strain LBE2040 ($A_S B_S$ *fus*) and selecting for tetracycline resistance. A high frequency of transductants showed co-transduction of fusidic acid sensitivity, indicating that the Tc^R gene was linked to the sensitive *fus* gene of LZ18L, which is located upstream of *tufA* in the *str* operon. After identification of the *tufA* mutation, the same P1 lysate was used to simultaneously transduce *rpsL666 tufA_R* (a 90% linkage exists between these two genes) to strains LBE2041, EV102, LBE2020 and PM505 to obtain the isogenic strains, AZ25L, AZ27L, AZ29L and AZ31L, respectively, differing only in their *tufB* allele. The strains used were (1) selected for tetracycline resistance, because the Tc^R gene is linked to *tufA_R* in LZ18L, and (2) screened for small colonies, because strains harbouring *rpsL666* and *tufA_R* grow more slowly than strains harbouring only *tufA_R*. Strain AZ1 was constructed by transducing UA254 (*miaA*) with a P1 lysate of UA244 (*rpsL282*) and selecting for Sm^D colonies on LC plates containing 100 μg streptomycin ml^{-1} . Screening for kirromycin resistance was done on plates containing 1.5 mM EDTA (pH 8.0) and 100 μg kirromycin ml^{-1} . Kirromycin was a generous gift from Gist-Brocades.

Growth rates were determined at 37 °C in 10 ml liquid LC medium by measuring the time required for a doubling of the OD_{560} during exponential growth phase. Cultures were grown

Table 1. *E. coli* strains used in this study

Strain*	Genotype	Reference
AZ1	<i>rpsL282 miaA rpoB valR gyrA thi trpT</i> (Su9) <i>pur</i> ⁺ Δ (<i>pro-lac</i>) (F' <i>lac pro Tn10dcat</i>)	This work
AZ25L	<i>rpsL666† tufA‡ sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
AZ27L	<i>rpsL666† tufA‡ tufB‡ sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
AZ29L	<i>rpsL666† tufA‡ tufB§ sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
AZ31L	<i>rpsL666† tufA‡ tufB sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
CM1234	<i>rpsL827¶ sup</i> ^o (F' <i>lac pro Tn10dcat</i>)	Timms & Bridges (1993)
CM1243	<i>rpsL833¶ sup</i> ^o (F' <i>lac pro Tn10dcat</i>)	Timms & Bridges (1993)
EV102	<i>tufB‡ sup</i> ^o <i>rpoB fus</i> (F' <i>lac pro Tn10dcat</i>)	Vijgenboom & Bosch (1987)
LBE2020	<i>tufB§ sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	Van der Meide <i>et al.</i> (1982)
LBE2040	<i>sup</i> ^o <i>fus</i>	Van de Klundert <i>et al.</i> (1978)
LBE2041	<i>sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	Van de Klundert <i>et al.</i> (1978)
LZ18L	<i>rpsL666† tufA‡ sup</i> ^o (F' <i>lac pro Tn10dcat</i>)	This work
LZ50	<i>rpsL666† tufB::Mu sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
LZ52L	<i>rpsL666† tufA‡ tufB::Mu sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	This work
PM505	<i>tufB::Mu sup</i> ^o <i>rpoB</i> (F' <i>lac pro Tn10dcat</i>)	Van der Meide <i>et al.</i> (1982)
UA244	<i>rpsL282 rpoB valR gyrA thi trpT</i> (Su9) <i>aro</i> ⁺ Δ (<i>pro-lac</i>)	Faxén <i>et al.</i> (1988)
UA254	<i>miaA rpoB valR gyrA thi trpT</i> (Su9) <i>pur</i> ⁺ Δ (<i>pro-lac</i>) (F' <i>lac pro Tn10dcat</i>)	Faxén <i>et al.</i> (1988)
UD666	<i>rpsL666† sup</i> ^o	Ruusala <i>et al.</i> (1984)

* Strains that end with L are Tc^R M13mp9Zam20C lysogens.

† The *rpsL* mutation is P90L.

‡ The *tuf* mutation is A375T.

§ The *tufB* mutation is G222D.

|| The *rpsL* mutation is K42Q.

¶ The *rpsL* mutation is P90R.

in 100 ml flasks in a gyratory water-bath shaker (New Brunswick Scientific). The values in Table 2 are means of three independent measurements.

PCR and sequencing. For PCR amplification of chromosomal DNA from strains AZ25L, AZ27L, AZ29L, AZ31L and UD666, cells from a 10 ml overnight culture were washed with 3 ml H₂O and resuspended in 250 μ l H₂O. The resuspended pellet was boiled for 10 min and the cell debris was pelleted at 13000 r.p.m. for 5 min; 5 μ l of the supernatant was added to a mixture containing standard PCR buffer and primers. Pfu polymerase (Stratagene) was used to sustain the PCR reaction. The DNA oligonucleotides used for *rpsL* amplification were Bio733 5' CCAGGAGCTCTTTAATGGCAACAG 3' (nt -14 to +10 of *rpsL*, creating a *SacI* site) and Bio642 5' GGCGCACGTTTTTCGAGAGCTACTT 3' (nt +211 to +188 of *rpsG*, about 200 bp downstream of a *Bam*HI site). The PCR product (about 700 bp) was digested with *SacI* and *Bam*HI and cloned into pUC18. The *rpsL* fragment was sequenced by the dideoxy method using T7 DNA polymerase (Pharmacia) and [α -³²P]dCTP (ICN).

Kirromycin band-shift assay. Strains were grown to an OD₅₆₀ of 0.5 in 50 ml LC medium containing 50 μ g tetracycline ml⁻¹. The cells were harvested, washed and resuspended in 500 μ l standard buffer (50 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 40 mM NH₄Cl, 1 mM DTT). The cells were disrupted by sonication and the cell debris was removed at 13000 r.p.m. for 30 min, yielding a cell-free extract. The ribosomes were subsequently removed by centrifugation of the cell-free extract at 100000 g in an Airfuge (Beckman) for 20 min; 2.5 μ l of the

ribosome-free extract was diluted in standard buffer with 10% (v/v) glycerol in the presence or absence of 50 μ M kirromycin (final volume 25 μ l) and incubated at 0 °C for 10 min. Samples of the reaction mixtures (5 μ l) were electrophoresed on a 12% (v/v) non-denaturing polyacrylamide gel as described by Anborgh & Parmeggiani (1991), blotted onto nitrocellulose and probed with antibodies raised against EF-Tu.

Poly(U)-directed poly(Phe) synthesis. Strain AZ27L was used to prepare an S30 extract containing Sm^D ribosomes from which the endogenous EF-Tu was removed by immunoretaention (Talens *et al.*, 1996). Wild-type EF-Tu and EF-TuA[375T] were purified as described by Mesters *et al.* (1994). EF-Tu[G222D] has a C-terminal Ser(His)₆ extension, which enables purification by Ni²⁺-affinity chromatography (Boon *et al.*, 1992). Purified EF-Tu was added to reaction mixtures (50 μ l) containing 8 μ l AZ27L S30 extract, 40 mM Tris/acetate, pH 7.6, 10 mM magnesium acetate, 70 mM NH₄Cl, 1 mM DTT, 5 mM phosphoenolpyruvate, 2 mM ATP, 50 μ M GTP, 10 μ g pyruvate kinase ml⁻¹, 0.4 mg bulk tRNA (Sigma) ml⁻¹, 160 μ g poly(U) ml⁻¹ (Boehringer) and 2 μ l [¹⁴C]-Phe (50 μ Ci ml⁻¹, 150 mCi mmol⁻¹, 1 Ci = 3.7 $\times$ 10¹⁰ Bq) (ICN). Reactions were incubated at 37 °C for 10 min and stopped by the addition of 60 μ l 0.1 M NaOH and further incubation at 37 °C for 5 min. Polypeptides were precipitated by the addition of 5% (w/v) TCA and filtered on GF/C (Whatmann) filters. The amount of [¹⁴C]-Phe incorporated into polypeptides was determined by liquid-scintillation counting. Representative results from at least two experiments are given in Figs 1–3. The error range for the data points in Fig. 1 is up to 20%

for the smaller values, in Fig. 2 up to 17% and in Fig. 3. up to 25% (see error bars).

RESULTS

Identification of the Sm^D mutation in strain UD666

Although ribosomes isolated from *E. coli* strain UD666 (harbouring the *rpsL666* allele) have been used to analyse the functional defects of the Sm^D S12 mutation (Ruusala *et al.*, 1984), the mutation itself had not been identified. Sm^D mutations are known to cluster around position 42 and 90 in S12 and ancillary mutations in S12 frequently occur. The latter do not always cause a distinct phenotype but some improve the growth rate (Timms & Bridges, 1993).

The chromosomal *rpsL* gene from the Sm^D strain UD666 was amplified by PCR and sequenced. A C → T mutation was found, resulting in a change from proline to leucine at position 90 in the S12 protein. The same mutation had already been described in an *E. coli* B/r strain (Timms *et al.*, 1992; GenBank accession no. 147892). Sequencing the whole *rpsL* gene revealed no ancillary mutations.

Reversion of streptomycin dependence by mutant EF-Tu

In search of EF-Tu-specific suppressors of streptomycin dependence caused by mutations in S12, we used the technique of M13-mediated gene replacement to randomly mutagenize the *tuf* genes in the Sm^D strain UD666, followed by selection for streptomycin independence. P1 phage transduction and M13 retrieval showed that the *tufA* gene was mutated and DNA sequence analysis revealed the A375T (A_R) mutation in EF-Tu. This mutation had previously been identified as a kirromycin-resistant and error-prone mutation (Duisterwinkel *et al.*, 1981; Bosch *et al.*, 1996 and references therein). To exclude the possibility that suppression of streptomycin dependence was due to other mutation(s) in the M13 phage, strain UD666 was

infected with an M13 lysate containing solely *tufA_R*. Again we found suppression of streptomycin dependence, indicating that EF-TuA_R is indeed responsible for reversion of the Sm^D phenotype to an independent (Sm^I) and even streptomycin-sensitive (Sm^S) phenotype. Other kirromycin-resistant EF-Tu species, with mutations such as Q124K, also error-prone, G316D or A375V (Mesters *et al.*, 1994), were not able to reverse the streptomycin dependence of strain UD666. Repeated attempts to suppress the Sm^D phenotype of UD666 by introducing the A375T mutation in *tufB* were unsuccessful. This may be due to the lower expression from the *tufB* gene (van der Meide *et al.*, 1982). We also tested whether other Sm^D S12 mutations could be suppressed by EF-TuA_R. *tufA_R* was introduced using M13-mediated allelic replacement in strains CM1234 and CM1243, containing the S12 mutations K42Q and P90R, respectively. In both cases the presence of EF-TuA_R reversed the Sm^D phenotype to Sm^I. However, streptomycin dependence of strain AZ1 caused by the double mutation *miaA/rpsL282* could not be suppressed by the A_R mutation.

In *rpsL*⁺ strains kirromycin sensitivity is dominant over resistance (van de Klundert *et al.*, 1978), but here the Sm^I strain LZ18L (*rpsL666 tufA_R*) turned out to be kirromycin-resistant, despite the presence of wt EF-TuB. This phenomenon has already been observed in *Salmonella typhimurium* harbouring EF-Tu[A375T] and an error-restrictive Sm^R mutation in S12 (Tubulekas *et al.*, 1991).

The effect of mutant EF-TuB species in the Sm^I strain

The Sm^I strain LZ18L contains a mixed EF-Tu population, EF-TuA_R and wt EF-TuB (B_S). This raised the question whether the combination of these two different EF-Tu species is needed for suppression of streptomycin dependence or whether EF-TuA_R alone would be sufficient. We therefore tried to isolate Sm^I colonies after randomly mutagenizing *tufA* in an Sm^D strain with

Table 2. Growth rate of strains harbouring mutations in *rpsL*, *tufA* and/or *tufB*

Strain	Relevant genotype	Relevant phenotype	Doubling time (min)	Relative doubling time (%)
AZ25L	<i>rpsL666 tufA_R tufB_S</i>	Sm ^I	98 ± 2	1·00
AZ27L	<i>rpsL666 tufA_R tufB_R</i>	Sm ^I	95 ± 3	0·97
AZ29L	<i>rpsL666 tufA_R tufB₀</i>	Sm ^I	105 ± 2	1·07
AZ31L	<i>rpsL666 tufA_R tufB::Mu</i>	Sm ^I	129 ± 4	1·32
PM816	<i>rpsL⁺ tufA_R tufB_S</i>	Sm ^S	30*	1·00
—	<i>rpsL⁺ tufA_R tufB_R</i>	Sm ^S	ND	—
LBE2021	<i>rpsL⁺ tufA_R tufB₀</i>	Sm ^S	31*	1·03
PM455	<i>rpsL⁺ tufA_R tufB::Mu</i>	Sm ^S	28*	0·93

ND, Not determined by Van der Meide *et al.* (1982).

* Values from van der Meide *et al.* (1982).

an insertionally inactivated *tufB* (LZ50), but several attempts were unsuccessful. However, when an M13 lysate containing *tufA_R* was used, an Sm^I strain (LZ52L) was isolated, but only after long incubation (72 h) and with a low transduction frequency. Thus, EF-Tu_{A_R} alone is capable of suppressing streptomycin dependence, but not as well as when wt EF-TuB is present. Is this due to a reduced cellular concentration of EF-Tu in the cell or is a cooperative effect between EF-Tu_{A_R} and wt EF-TuB responsible for good complementation? To answer this question a series of isogenic strains was constructed, harbouring *rpsL666 tufA_R* and different alleles of *tufB*. *rpsL666* and *tufA_R* were simultaneously transduced to strains LBE2041 (A_SB_S), EV102 (A_SB_R), LBE2020 (A_SB₀) and PM505 (A_SB::Mu) because manipulating them separately proved to be difficult due to a 90% linkage between the two genes. It appeared that the resulting strains, AZ25L, AZ27L, AZ29L and AZ31L, easily generated revertants and we were very careful in selecting and checking colonies for further experiments. The nature of EF-TuA and EF-TuB in these strains was confirmed using a kirromycin band-shift assay. This assay is based on the difference in electrophoretic mobility under non-denaturing conditions of EF-Tu bound to kirromycin as compared to unbound EF-Tu. EF-Tu_{A_R} does not bind kirromycin because of its resistant character and can thus be distinguished from wt EF-Tu and EF-TuB₀ in this assay (data not shown). The presence of the P90L mutation in S12 of the various strains was confirmed by sequencing their *rpsL* genes. This also showed that no ancillary mutations in *rpsL* had occurred during the manipulation of these strains.

Growth rate measurements in rich medium clearly demonstrated that the combination of the two different EF-Tu species, A_R and B_S, is not the only possibility for good reversion of streptomycin dependence (Table 2). The latter is also achieved when wt EF-TuB is replaced by EF-TuB_R or EF-TuB₀. Furthermore, the growth rate of Sm^I *rpsL666 tufA_R* strains is more susceptible than that of the corresponding *rpsL*⁺ strains to reduction in the EF-Tu concentration, since the generation time of strain AZ31L carrying a *tufB* disruption is increased by about 30% (Table 2, compare strains AZ25L and AZ31L). In *rpsL*⁺ strains this disruption does not lead to a reduction in growth rate in rich medium (Table 2, compare strains PM816 and PM455) (van der Meide *et al.*, 1982).

In vitro analysis of streptomycin dependence suppression by mutant EF-Tu species

EF-Tu[G222D] (B₀) alone is not able to sustain *in vitro* protein synthesis with wt ribosomes. However, *in vivo* a positive effect of EF-Tu[G222D] on growth rate was observed in the presence of Sm^D ribosomes and EF-Tu[A375T] (Table 2). We therefore also tested *in vitro* the ability of wt EF-Tu, EF-Tu[A375T] and EF-Tu[G222D] to sustain protein synthesis in an EF-Tu-depleted S30 extract of strain AZ27L containing Sm^D

Fig. 1. Poly(U)-directed poly(Phe) synthesis with different EF-Tu species and Sm^D ribosomes. Purified wt EF-Tu (◆), EF-Tu[A375T] (■) or EF-Tu[G222D] (▲) were added at the indicated concentrations to an EF-Tu-depleted S30 extract of AZ27L and their translational activity was measured. For further experimental details, see Methods.

Fig. 2. Poly(U)-directed poly(Phe) synthesis with Sm^D ribosomes as a function of Mg²⁺ concentration. Activity in poly(Phe) synthesis of 0.5 μM wt EF-Tu (◆), 0.5 μM EF-Tu[A375T] (■), 0.5 μM EF-Tu[G222D] (▲) or no EF-Tu (○) was measured as described in Methods.

ribosomes (Fig. 1). In the absence of streptomycin, wt EF-Tu is still active in the poly(Phe) synthesis assay, but about 60% less so than EF-Tu[A375T], while EF-Tu[G222D] is not active at all. Furthermore, the activities of wt EF-Tu and EF-Tu[A375T] appear to be optimal at 9–10 mM Mg²⁺, while EF-TuB[G222D] is weakly active only at non-physiological Mg²⁺ concentrations of 14 mM and higher (Fig. 2). Thus, EF-Tu[G222D] alone is not likely to productively interact with Sm^D ribosomes *in vivo*.

Fig. 3. Poly(U)-directed poly(Phe) synthesis with Sm^D ribosomes and EF-Tu_R and the stimulation by other EF-Tu species. Activity in poly(Phe) synthesis of 0.3 μM wt EF-Tu (◆), 0.3 μM EF-Tu[G222D] (▲), 0.3 μM EF-Tu[A375T] (■), no EF-Tu (○) or combinations of 0.3 μM wt EF-Tu/0.3 μM EF-Tu[A375T] (◇) and 0.3 μM EF-Tu[G222D]/0.3 μM EF-Tu[A375T] (△) was measured as a function of time by using the reaction mixtures and incubations as described in Methods. Error bars are given for two independent measurements.

Synergism between EF-Tu[A375T] and EF-Tu[G222D] could be another explanation for our *in vivo* results, since such an effect had already been observed *in vivo* for their joint stimulation of read-through and frame-shifting. To examine this possibility we analysed the activities of wt EF-Tu or EF-Tu[G222D] in combination with EF-Tu[A375T] and the isolated activities in the poly(Phe) synthesis assay with Sm^D ribosomes at 10 mM Mg^{2+} . No synergistic effect could be observed when either wt EF-Tu or EF-Tu[G222D] was added to EF-Tu[A375T] in the reaction mixture (Fig. 3). In the latter combination we found exactly the same activity as for EF-Tu[A375T] alone.

DISCUSSION

Reversion of streptomycin dependence

Streptomycin dependence caused by mutations in ribosomal protein S12 can be complemented by *ram* mutations in ribosomal proteins S4 and S5, and in 16S rRNA (Birge & Kurland, 1970; Deusser *et al.*, 1970; Allen & Noller, 1991; Lodmell & Dahlberg, 1997). Here we have shown that the kirromycin-resistant and error-prone EF-Tu with mutation A375T (EF-TuA_R) is also able to complement the Sm^D phenotype of different S12 mutations. However, this is not a general property of kirromycin-resistant EF-Tus, since suppression of streptomycin dependence by EF-Tu[Q124K] or EF-Tu[G316D] could not be demonstrated. Furthermore,

EF-Tu[A375T] is not able to reverse streptomycin dependence caused by the double mutation *miaA/rpsL282*, suggesting that this double mutation belongs to a class of Sm^D mutations different from the above-mentioned S12 mutations. A subdivision of Sm^D mutations was reported by Bjare & Gorini (1971).

Recently, it became evident that a conformational switch in the 912 region of 16S rRNA is involved in translational fidelity (Lodmell & Dahlberg, 1997). The two alternating base-paired arrangements produce either an error-prone or a restrictive state. Restrictive mutations in this switch region are compatible with *ram* mutations in S5. On the other hand, error-prone mutations in the switch region are compatible with restrictive mutations in S12. Ribosomal proteins S5 and S12 are probably in close contact with the 912 region and facilitate switching between the two conformations. The EF-Tu binding site on the ribosome is in the proximity of S12 and not far from the 912 region. Our findings therefore fit the model by assuming that the error-prone character of the A375T mutation in EF-Tu disturbs switching between the two conformers (either directly or via S12) at a certain stage during initial codon-anticodon recognition or proofreading, and that this is counteracted by a restrictive mutation in S12. The kirromycin-resistant and also error-prone EF-Tu[Q124K] mutation (previously described as EF-TuA^{*}; Vijgenboom & Bosch, 1989) did not reverse streptomycin dependence. Thus, suppression of the Sm^D phenotype is not simply a matter of balancing translational accuracy, but probably requires specific interactions between EF-Tu and S12. Codons 40–43 and 87–90 in S12 are known to be hot spots for Sm^D mutations (Timms *et al.*, 1992). We found that not only strains harbouring the Sm^D mutation P90L, but also strains harbouring the Sm^D mutations P90R or K42Q become Sm^I after expression of EF-TuA_R. Unfortunately, the three-dimensional structure of S12 is not known, but one could imagine that positions 42 and 90 are in close proximity as part of a contact area controlling translational accuracy.

Strains carrying both the Sm^D *rpsL666* and the *tufA_R* mutations show a reversion to streptomycin sensitivity. In addition, these strains have become kirromycin-resistant although sensitive wt EF-Tu is present. Such a dominant kirromycin-resistant phenotype, together with a reduced resistance to streptomycin, has also been described for an *S. typhimurium* strain harbouring an error-restrictive Sm^R S12 mutation in combination with the EF-Tu[A375T] mutation (Tubulekas *et al.*, 1991). For these hyperaccurate ribosomes it has been shown that they preferentially use EF-Tu[A375T] for translation. The same may also be true for Sm^D ribosomes, because under non-saturating conditions EF-Tu[A375T] is 2.5-fold more active in poly(Phe) synthesis than wt EF-Tu when these ribosomes are used. On the other hand, we previously reported that EF-Tu[A375T] is threefold less active than wt EF-Tu with wt ribosomes (Talens *et al.*, 1996).

Cell growth enhancement by EF-TuB

Although EF-TuA_R suppresses the Sm^D phenotype of strain UD666, the generation time of Sm^I strains is always three- to fourfold longer than that of the corresponding *rpsL*⁺ strain, indicating that not all defects caused by the S12 mutation can be complemented by EF-TuA_R. Furthermore, the expression of wt EF-Tu, EF-TuB_R, or EF-TuB₀ from a *tufB* allele in strains harbouring *rpsL666 tufA_R* (AZ25L, AZ27L and AZ29L, respectively) increased the growth rate to the same extent when compared to the isogenic strain lacking EF-TuB (AZ31L). An explanation could be that the presence of wt EF-TuB is already sufficient for maximal suppression of the Sm^D phenotype *in vivo*, although less effective at limiting concentrations in sustaining poly(Phe) synthesis with Sm^D ribosomes than EF-Tu[A375T]. *In vitro*, we observe an additive effect of wt EF-Tu on protein synthesis with Sm^D ribosomes in combination with EF-Tu[A375T] which might account for the increased growth rate of such a strain in comparison to a *tufB*::Mu strain.

The result with EF-TuB₀ is more puzzling because this mutant EF-Tu species is not active in protein synthesis with wt ribosomes. The main effect of the G222D mutation is an inhibition of the signal transmission to the GTPase centre of EF-Tu triggered by codon-induced conformational transitions of the tRNA (Vorstenbosch *et al.*, 1996). Normally, the transmission leads to GTP hydrolysis and further steps of A-site binding. This inhibition can be overcome by increasing the amount of Mg²⁺. We tested whether EF-Tu[G222D] would have a better functional interaction with Sm^D ribosomes, but, just as with wt ribosomes, EF-Tu[G222D] was inactive in poly(Phe) synthesis at physiological Mg²⁺ concentrations (5–9 mM) and became active only at higher Mg²⁺ concentrations. The effect on the growth rate could also be due to a sort of synergism between EF-Tu[A375T] and EF-Tu[G222D] as reported for *in vivo* suppression of nonsense and frameshift mutations (Vijgenboom *et al.*, 1985; Vijgenboom & Bosch, 1989). However, under our *in vitro* conditions no such synergistic contribution of EF-Tu[G222D] to protein synthesis could be detected.

Therefore, the positive contribution of EF-TuB₀ in suppressing streptomycin dependence together with EF-TuA_R as seen in growth-rate experiments, awaits further explanation. In cells with active *tufA* and *tufB*, the EF-Tu/aa-tRNA ratio is about 1:1 and complex formation is advantageous for the protection of the labile aminoacyl ester bond against unwanted hydrolysis. EF-TuB₀ is not disturbed in ternary complex formation and thus protects a certain amount of aa-tRNA in the cell from spontaneous deacylation. It cannot deliver the aa-tRNA to the ribosomal A-site, but it could perhaps act as a supplier of aa-tRNA to EF-Tu species which are active in protein synthesis, if we assume that a rapid transfer of aa-tRNA from EF-TuB₀·GTP·aa-tRNA to, for example EF-TuA_R·GTP, is possible. This might then explain the positive contribution of EF-TuB₀ to

suppression of streptomycin dependence as compared to strains lacking EF-TuB.

The abundance of EF-Tu in fast-growing cells (8–10 copies per ribosome) has promoted searches for other roles for EF-Tu. Several groups have speculated about an additional role connected to the reported additional round of GTP hydrolysis per peptide bond formation (Weijland & Parmeggiani, 1993; Ehrenberg *et al.*, 1993; Bosch *et al.*, 1996). Recently, a chaperone-like refolding activity was reported for EF-Tu (Kudlicki *et al.*, 1997). The positive contribution of the defective EF-TuB₀ to the Sm^I phenotype described in this paper could well be understood from its participation, just like wt EF-Tu, in such an additional and vital role.

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