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

Anne-Marie Zuurmond, Leo A. H. Zeeff† and Barend Kraal

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, and known to confer a kirromycin-resistant, error-prone phenotype. Also, other streptomycin-dependent (SmD) S12 mutations could be complemented by this mutation. The streptomycin-independent (SmI) strain grows more slowly than the wild-type (wt), suggesting that not all the defects of the S12 mutation can be complemented by EF-TuA. 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-TuA but, remarkably, also of EF-TuB, known as EF-TuB and defective in protein synthesis, equally contributed to cell growth. In vitro analysis revealed a decreased translational activity of wt EF-Tu with SmD ribosomes as compared to EF-TuA, while EF-TuB showed no activity at all, just as with wt ribosomes. Possible mechanisms are discussed for the improved growth rate observed in such SmI strains when they include wt EF-Tu or EF-TuB.

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 a-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,
1991 and references therein), but also by mutations in ribosomal protein S12 (Ozaki et al., 1969). Streptomycin-resistant (SmR) 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 (SmR) mutations are clustered in two regions, one spanning codons 40-43, the other 87-93 (Timms et al., 1992). The SmR phenotype is not caused by hyperaccuracy alone since some of the SmR mutants that do not require streptomycin are more error-restrictive than SmR mutants (Ruusala et al., 1984; Andersson et al., 1986). Quench-flow analysis has shown that SmR ribosomes, in addition to the defects they have in common with SmR ribosomes, are perturbed in a step between peptidyl transfer and translational 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 SmR 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 SmR S12 mutation with a mutA 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 SmR 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-TuAR (A375T), EF-TuA (later identified as Q124K) and the null mutant EF-TuB (G222D), which were originally found in kirromycin-resistant strains (Duisterwinkel et al., 1981, 1984; Zeef & Bosch, 1993) and promote frameshifting (Hughes et al., 1987; Vigenboom & Bosch, 1989). The subscripts in A, and B, refer to their wild-type (wt) cells with EF-TuA, and EF-TuB, (for further explanation, see van der Meide & Vigenboom, 1981). When EF-TuAR or EF-TuA is combined with EF-TuB, the frequency of frameshifting exceeds the sum of the separate contributions (Vigenboom & Bosch, 1989). Such a synergistic effect between EF-TuAR and EF-TuB has also been observed for nonsense suppression (Vigenboom et al., 1985). The increase in frameshifting and nonsense suppression caused by EF-TuB 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 accuracy 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-TuAR mutation can also cause suppression of streptomycin dependence. This mutation has been reported to complement growth defects of hyperaccurate SmR 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 SmR strain UD666 (harbouring the rpsL666 allele) were subjected to random mutagenesis by M13-mediated allelic replacement (Zeeef & Bosch, 1993). A tetracycline-resistant (TcR) 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 ng streptomycin ml-1, followed by incubation at 37°C for 2 h after removal of the unadsorbed phages. Selection for streptomycin-independent (Sm) strains was on LC plates (Zeeef & Bosch, 1993) containing 50 ng tetracycline ml-1 and lacking streptomycin. To identify the tuf mutation in the Sm strain LZ18L, M13 retrieval was performed. Since retrieval of the mutation would make the strain SmR 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 SmR strain sensitive to streptomycin. The mutation was identified by sequencing the retrieved M13 using T7 DNA polymerase (Pharmacia), [a32P]dCTP (ICN) and tuf primers (Zeeef & 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 fusicidic-acid-resistant strain LBE2040 (A, fus) and selecting for tetracycline resistance. A high frequency of transductants showed co-transduction of fusicidic acid sensitivity, indicating that the TcR gene was linked to the sensitive fus gene of LZ18L, which is located upstream of tufA in the str operon. After identification of the tuf mutation, the same P1 lysate was used to simultaneously transduce rpsL666 tufAR (a 90% linkage exists between these two genes) to strains LBE2041, EV102, LBE2020 and PM505 to obtain the isogenic strains, A22L, A22L, A22L and A231L, respectively, differing only in their tufB allele. The strains used were (1) selected for tetracycline resistance, because the Tc gene is linked to tufA in LZ18L, and (2) screened for small colonies, because strains harboring rpsL666 and tufAR grow more slowly than strains harbouring only tufAR. Strain AZ1 was constructed by transducing UA2044 (miaA) with a P1 lysate of UA244 (rpsL282) and selecting for SmR 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 OD650 during exponential growth phase. Cultures were grown
**Table 1. E. coli strains used in this study**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ1</td>
<td>rpsL282 miaA rpoB valR gyrA thi trpT (Su9) pur+ Δ(pro-lac) (F'lac pro Tn10dcat)</td>
<td>This work</td>
</tr>
<tr>
<td>AZ25L</td>
<td>rps666+ tufA+ sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>This work</td>
</tr>
<tr>
<td>AZ27L</td>
<td>rps666+ tufA+ sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>This work</td>
</tr>
<tr>
<td>AZ29L</td>
<td>rps666+ tufA+ sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>This work</td>
</tr>
<tr>
<td>AZ31L</td>
<td>rps666+ tufA+ sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>This work</td>
</tr>
<tr>
<td>CM1234</td>
<td>sup+ (F'lac pro Tn10dcat)</td>
<td>Timms &amp; Bridges (1993)</td>
</tr>
<tr>
<td>CM1243</td>
<td>sup+ (F'lac pro Tn10dcat)</td>
<td>Timms &amp; Bridges (1993)</td>
</tr>
<tr>
<td>EV102</td>
<td>sup+ rpoB fus (F'lac pro Tn10dcat)</td>
<td>Van der Meide et al. (1982)</td>
</tr>
<tr>
<td>LBE2020</td>
<td>sup+ fus</td>
<td>Van de Klundert et al. (1978)</td>
</tr>
<tr>
<td>LBE2040</td>
<td>sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>Van de Klundert et al. (1978)</td>
</tr>
<tr>
<td>LBE2041</td>
<td>sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>This work</td>
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<td>This work</td>
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<td>This work</td>
</tr>
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<td>PM505</td>
<td>sup+ rpoB (F'lac pro Tn10dcat)</td>
<td>Van der Meide et al. (1982)</td>
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<td>UA244</td>
<td>rpsL282 rpoB valR gyrA thi trpT (Su9) aro+ Δ(pro-lac)</td>
<td>Faxén et al. (1988)</td>
</tr>
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<td>UA254</td>
<td>miaA rpoB valR gyrA thi trpT (Su9) pur+ Δ(pro-lac) (F'lac pro Tn10dcat)</td>
<td>Faxén et al. (1988)</td>
</tr>
<tr>
<td>UD666</td>
<td>rpsL666+ sup+</td>
<td>Ruusala et al. (1984)</td>
</tr>
</tbody>
</table>

* Strains that end with L are Te<sup>a</sup> M13mp9Zam20C lysogens.
† The rps<sup>L</sup> mutation is P90L.
‡ The tuf mutation is A375T.
§ The tufB mutation is G222D.
|| The rps<sup>L</sup> mutation is K42Q.
¶ The rps<sup>L</sup> 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 AZ23L, AZ27L, AZ29L, AZ31L and UD666, cells from a 10 ml overnight culture were washed with 3 ml H<sub>2</sub>O and resuspended in 250 μl H<sub>2</sub>O. The resuspended pellet was boiled for 10 min and the cell debris was pelleted at 13000 r.p.m. for 5 min; 5 μl 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 independent measurements. The DNA oligonucleotides used for rps<sup>L</sup> amplification were Bio733: 3' CCAGGACGTCTTTAATGGCAACAG 3' (nt −14 to +10 of rps<sup>L</sup>, creating a SacI site) and Bio642: 5' GGGCGAACGTCTTTCCAGAGCTACTT 3' (nt +211 to +188 of rpsG, about 200 bp downstream of a BamHI site). The PCR product (about 700 bp) was digested with SacI and BamHI and cloned into pUC18. The rps<sup>L</sup> fragment was sequenced by the dideoxy method using T7 DNA polymerase (Pharmacia) and [α-<sup>32</sup>P]dCTP (ICN).

**Kirromycin band-shift assay.** Strains were grown to an OD<sub>560</sub> of 0.5 in 50 ml LC medium containing 50 μg tetracycline ml<sup>−1</sup>. The cells were harvested, washed and resuspended in 500 μl standard buffer (50 mM Tris/ HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 40 mM NH<sub>4</sub>Cl, 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 100 000 g in an Airfuge (Beckman) for 20 min; 2.5 μl of the ribosome-free extract was diluted in standard buffer with 10% (v/v) glycerol in the presence or absence of 50 μM kirromycin (final volume 25 μl) and incubated at 0 °C for 10 min. The reaction mixtures (5 μl) 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<sup>R</sup> ribosomes from which the endogenous EF-Tu was removed by immunoretenion (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)<sub>14</sub> extension, which enables purification by Ni<sup>2+</sup>-affinity chromatography (Boon et al., 1992). Purified EF-Tu was added to reaction mixtures (50 μl) containing 8 μl AZ27L S30 extract, 40 mM Tris/acetate, pH 7.6, 10 mM magnesium acetate, 70 mM NH<sub>4</sub>Cl, 1 mM DTT, 5 mM phosphoenolpyruvate, 2 mM ATP, 50 μM GTP, 10 μg pyruvate kinase ml<sup>−1</sup>, 0.4 mg bulk tRNA (Sigma) ml<sup>−1</sup>, 160 μg poly(U) ml<sup>−1</sup> (Boehringer) and 2 μl C-Phe (50 μCi ml<sup>−1</sup>, 150 μCi mmol<sup>−1</sup>, 1 Ci = 3.7 × 10<sup>10</sup> Bq) (ICN). Reactions were incubated at 37 °C for 10 min and stopped by the addition of 60 μl 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 C-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<sup>6</sup> mutation in strain UD666

Although ribosomes isolated from <i>E. coli</i> strain UD666 (harbouring the <i>rpsL666</i> allele) have been used to analyse the functional defects of the Sm<sup>D</sup> S12 mutation (Ruusala et al., 1984), the mutation itself had not been identified. Sm<sup>D</sup> 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 <i>rpsL</i> gene from the Sm<sup>D</sup> 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 <i>E. coli</i> B/r strain (Timms et al., 1992; GenBank accession no. 147892). Sequencing the whole <i>rpsL</i> 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 <i>tuf</i> genes in the Sm<sup>6</sup> strain UD666, followed by selection for streptomycin independence. PI phage transduction and M13 retrieval showed that the <i>tuf</i>A gene was mutated and DNA sequence analysis revealed the A375T (AR) mutation in position 42 and 90 in S12 and ancillary mutations in S12. The A375T mutation had already been described in an EF-Tu-specific suppressor of streptomycin dependence caused by mutations in S12, we used the technique of M13-mediated gene replacement to randomly mutagenize the <i>tuf</i> genes in the Sm<sup>6</sup> strain UD666, followed by selection for streptomycin independence. PI phage transduction and M13 retrieval showed that the <i>tuf</i>A gene was mutated and DNA sequence analysis revealed the A375T (AR) mutation in position 42 and 90 in S12 and ancillary mutations in S12. The A375T mutation had already been described in an

| Table 2. Growth rate of strains harbouring mutations in <i>rpsL</i>, <i>tufA</i> and/or <i>tufB</i> |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strain          | Relevant genotype | Relevant phenotype | Doubling time (min) | Relative doubling time (%) |
| AZ25L           | <i>rpsL666 tufA<sub>R</sub> tufB<sub>R</sub></i> | Sm<sup>6</sup> | 98 ± 2 | 1:00 |
| AZ27L           | <i>rpsL666 tufA<sub>R</sub> tufB<sub>R</sub></i> | Sm<sup>6</sup> | 95 ± 3 | 0:97 |
| AZ29L           | <i>rpsL666 tufA<sub>R</sub> tufB<sub>R</sub></i> | Sm<sup>6</sup> | 105 ± 2 | 1:07 |
| AZ31L           | <i>rpsL666 tufA<sub>R</sub> tufB<sub>R</sub> : Mu</i> | Sm<sup>6</sup> | 129 ± 4 | 1:32 |
| PM816           | <i>rpsL<sup>*</sup> tufA<sub>R</sub> tufB<sub>R</sub></i> | Sm<sup>6</sup> | 30* | 1:00 |
| LBE.2021        | <i>rpsL<sup>*</sup> tufA<sub>R</sub> tufB<sub>R</sub></i> | Sm<sup>6</sup> | ND | – |
| PM455           | <i>rpsL<sup>*</sup> tufA<sub>R</sub> tufB<sub>R</sub> : Mu</i> | Sm<sup>6</sup> | 28* | 0:93 |

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

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

The effect of mutant EF-TuB species in the Sm<sup>R</sup> strain

The Sm<sup>R</sup> strain LZ18L contains a mixed EF-Tu population, EF-Tu<sub>A</sub> and wt EF-TuB (B<sub>R</sub>). 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<sub>R</sub> alone would be sufficient. We therefore tried to isolate Sm<sup>R</sup> colonies after randomly mutagenizing <i>tuf</i>A in an Sm<sup>D</sup> strain with
Mutant EF-Tu reverses streptomycin dependence

an insertionally inactivated *tufB* (LZ50), but several attempts were unsuccessful. However, when an M13 lysate containing EF-TuAR was used, an Sm' strain (LZ52L) was isolated, but only after long incubation (72 h) and with a low transduction frequency. Thus, EF-TuAR 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-TuAR and wt EF-TuB responsible for good complementation? To answer this question a series of isogenic strains was constructed, harbouring *rpsL666* and different alleles of *tufB*. *rpsL666* and *tufAR* were simultaneously transduced to strains LBE2041 (A,B,), EV102 (A,B,), LBE2020 (A,B,) and PM505 (A,B::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 bandshift 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-TuAR does not bind kirromycin because of its resistant character and can thus be distinguished from wt EF-Tu and EF-TuB 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_R, 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-TuB_R. Furthermore, the growth rate of Sm' *rpsL666 tufAR* 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] (B_R) 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 SmD 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 SmD 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 Mg^{2+}, while EF-TuB[G222D] is weakly active only at non-physiological Mg^{2+} concentrations of 14 mM and higher (Fig. 2). Thus, EF-Tu[G222D] alone is not likely to productively interact with SmD ribosomes *in vivo*.

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**Fig. 1.** Poly(U)-directed poly(Phe) synthesis with different EF-Tu species and SmD 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 SmD ribosomes as a function of Mg^{2+} 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.
Fig. 3. Poly(U)-directed poly(Phe) synthesis with SmD ribosomes and EF-Tu, 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 frameshifting. 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 SmD ribosomes at 10 mM MgCl₂. 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; Deussel 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-TuAR) is also able to complement the SmD 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 SmD mutations different from the above-mentioned S12 mutations. A subdivision of SmD 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*; Vrijenboom & Bosch, 1989) did not reverse streptomycin dependence. Thus, suppression of the SmD 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 SmD mutations (Timms et al., 1992). We found that not only strains harbouring the SmD mutation P90L, but also strains harbouring the SmD mutations P90R or K42Q become SmΔ after expression of EF-TuAR. 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 SmD *rpsL666* and the *tufAR* 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 SmΔ 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 SmD 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\textsubscript{R} suppresses the Sm\textsuperscript{D} phenotype of strain UD666, the generation time of Sm\textsuperscript{I} strains is always three- to fourfold longer than that of the corresponding \textsc{rpsL}\textsuperscript{+} strain, indicating that not all defects caused by the S12 mutation can be complemented by EF-TuA\textsubscript{R}. Furthermore, the expression of wt EF-Tu, EF-TuB\textsubscript{R}, or EF-TuB\textsubscript{D} from a \textit{tuft} allele in strains harbouring \textsc{rpsL}666 EF-TuA\textsubscript{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\textsuperscript{D} phenotype \textit{in vivo}, although less effective at limiting concentrations in sustaining poly(Phe) synthesis with Sm\textsuperscript{D} ribosomes than EF-Tu[A375T]. \textit{In vitro}, we observe an additive effect of wt EF-Tu on protein synthesis with Sm\textsuperscript{D} ribosomes in combination with EF-Tu[A375T] which might account for the increased growth rate of such a strain in comparison to a \textit{tuft}::\textit{Mu} strain.

The result with EF-TuB\textsubscript{D} 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 Mg\textsuperscript{2+}. We tested whether EF-TuG222D would have a better functional interaction with Sm\textsuperscript{D} ribosomes, but, just as with wt ribosomes, EF-TuG222D was inactive in poly(Phe) synthesis at physiological Mg\textsuperscript{2+} concentrations (5–9 mM) and became active only at higher Mg\textsuperscript{2+} concentrations. The effect on the growth rate could also be due to a sort of synergism between EF-Tu[A375T] and EF-TuG222D as reported for \textit{in vivo} suppression of nonsense and frameshift mutations (Vijgenboom et al., 1985; Vijgenboom & Bosch, 1989). However, under our \textit{in vitro} conditions no such synergistic contribution of EF-TuG222D to protein synthesis could be detected.

Therefore, the positive contribution of EF-TuB\textsubscript{D} in suppressing streptomycin dependence together with EF-TuA\textsubscript{R} as seen in growth-rate experiments, awaits further explanation. In cells with active \textit{tuftA} and \textit{tuftB}, 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-TuB\textsubscript{D} is not disturbed in ternary complex formation and thus protects a certain amount of aa-tRNA in the cell from spontaneous decylation. 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-TuB\textsubscript{D} GTP aa-tRNA to, for example EF-TuA\textsubscript{R} GTP, is possible. This might then explain the positive contribution of EF-TuB\textsubscript{D} 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-Tu\textsubscript{D} to the Sm\textsuperscript{D} phenotype described in this paper could well be understood from its participation, just like wt EF-Tu, in such an additional and vital role.

ACKNOWLEDGEMENTS

We thank Professor L. Bosch (Leiden University) and Dr D. Hughes (Uppsala University) for their interest and for critical reading of the manuscript. This work is supported by the Netherlands Foundation for Chemical Research (SON 328-035) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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Received 24 April 1998; revised 11 August 1998; accepted 20 August 
1998.