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The Unique *tuf2* Gene from the Kirromycin Producer *Streptomyces ramocissimus* Encodes a Minor and Kirromycin-Sensitive Elongation Factor Tu

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***Streptomyces ramocissimus*, the producer of elongation factor Tu (EF-Tu)-targeted antibiotic kirromycin, contains three divergent *tuf*-like genes, with *tuf1* encoding regular kirromycin-sensitive EF-Tu1; the functions of *tuf2* and *tuf3* are unknown. Analysis of the *tuf* gene organization in nine producers of kirromycin-type antibiotics revealed that they all contain homologues of *tuf1* and sometimes of *tuf3* but that *tuf2* was found in *S. ramocissimus* only. The *tuf2*-flanking regions were sequenced, and the two *tuf2*-surrounding open reading frames were shown to be oriented in opposite directions. In vivo transcription analysis of the *tuf2* gene displayed an upstream region with bidirectional promoter activity. The transcription start site of *tuf2* was located approximately 290 nucleotides upstream of the coding sequence. Very small amounts of *tuf2* transcripts were detected in both liquid- and surface-grown cultures of *S. ramocissimus*, consistent with the apparent absence of EF-Tu2 in total protein extracts. The *tuf2* transcript level was not influenced by the addition of kirromycin to exponentially growing cultures. To assess the function of *S. ramocissimus* EF-Tu2, the protein was overexpressed in *Streptomyces coelicolor* LT2. This strain is a J1501 derivative containing His_6-tagged EF-Tu1 as the sole EF-Tu species, which facilitated the separation of EF-Tu2 from the interfering EF-Tu1. *S. ramocissimus* EF-Tu1 and EF-Tu2 were indistinguishable in their ability to stimulate protein synthesis in vitro and exhibited the same kirromycin sensitivity, which excludes the possibility that EF-Tu2 is directly involved in the kirromycin resistance mechanism of *S. ramocissimus*.**

Elongation factor Tu (EF-Tu), a member of the family of GTPase switch proteins, plays a pivotal role in the elongation process of bacterial protein synthesis (for a review see reference 15). The GTP-bound form of EF-Tu is responsible for delivery of aminoacyl-tRNA to the mRNA-programmed ribosomal A site. Cognate codon-anticodon recognition triggers the GTPase center on EF-Tu, causing the dissociation of inactive EF-Tu·GDP from the ribosome. Reactivation of the factor occurs via a nucleotide exchange reaction catalyzed by EF-Ts. EF-Tu is specifically affected by four different types of antibiotics of which kirromycin is the first identified and best studied (for references see reference 25). The binding of this antibiotic to EF-Tu still allows the factor to interact sequentially with aminoacyl-tRNA and the ribosomal A site. However, after GTP hydrolysis, EF-Tu·GDP is no longer ejected from the ribosome, thus immobilizing this and all following ribosomes on the mRNA, which explains the recessive character of kirromycin resistance in a mixed population of resistant and sensitive EF-Tu species. Certain error-restrictive mutations in ribosomal protein S12 (encoded by *rpsL*) overcome this recessivity; the mutant ribosomes will preferentially use the kirromycin-resistant EF-Tu for translation (33).

Polyketide antibiotic kirromycin and related compounds, called elfamycins, are produced by actinomycetes. These gram-positive mycelial soil bacteria undergo a complex process of morphological differentiation and produce a wide variety of secondary metabolites (12). Antibiotic production is generally confined to stationary phase in liquid culture and usually coincides with the onset of morphological differentiation in surface-grown cultures. Various mechanisms are exploited by antibiotic-producing microorganisms to protect themselves from the toxic action of their own products (5); these include the use of an efficient drug efflux system, intracellular storage of the antibiotic in an inactive form, modification of an otherwise sensitive target, and (temporary) expression of a resistant target. The mechanism used by producers of kirromycin-type antibiotics to protect themselves against their own products is only partially known; some producers contain an intrinsically kirromycin-resistant EF-Tu (4, 9).

Kirromycin producer *Streptomyces ramocissimus* contains three divergent *tuf* genes, which are designated *tuf1*, *tuf2*, and *tuf3* and which code for EF-Tus that are surprisingly heterogeneous: EF-Tu2 displays 88% amino acid identity with EF-Tu1, and EF-Tu3 shows only about 65% amino acid identity with both EF-Tu1 and EF-Tu2 (37). The *tuf1* gene encodes the major, kirromycin-sensitive EF-Tu (37) and is the promoter-distal gene in the *rpsL* operon, which also includes the genes for ribosomal proteins S12 (*rpsL*) and S7 (*rpsG*) and EF-G (*fus*). *tuf1* is transcribed at a very high level during exponential growth from both the *rpsL* operon promoter and a *tuf1*-specific promoter (32). The roles of *S. ramocissimus tuf2* and *tuf3* are not yet clear; the gene products could not be detected under normal growth conditions, and overexpression in *Escherichia coli* yielded inactive products, deposited in inclusion bodies (37). Studies of the genetically well-characterized *Streptomyces coelicolor* revealed that this strain contains both *tuf1* and *tuf3* homologues (35) but lacks a *tuf2* equivalent. Transcription of

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TABLE 1. Elfamycin-producing actinomycetes used in this study

Strain[a]	Antibiotic produced	*tuf* gene(s)	EF-Tu phenotypes[b] (reference)
S. cinnamoneus Tü89	Kirrothricin	*tuf1*	Kir^r (4, 9)
S. collinus Tü365	Kirromycin	*tuf1, tuf3*	Kir^s (9, 20)
S. diastatochromogenes Tü1062	Kirromycin	*tuf1*	Kir^s (9)
S. filipinensis NRRL 11044	Heneicomycin	*tuf1, tuf3*	Not known
S. fradiae Tü1222	Kirromycin	*tuf1, tuf3*	Kir^s (9)
S. goldiniensis ATCC 21386	Aurodox	*tuf1, tuf3*	Kir^s (9)
N. lactamdurans ATCC 27382	Efrotomycin	*tuf1*	Kir^r (9)
S. ramocissimus CBS 190.6	Kirromycin	*tuf1, tuf2, tuf3*	Kir^s (9, 37)
Streptomyces sp. strain NNRL 15496	SB22484 factors 1–4	*tuf1*	Not known

[a] *S., Streptomyces*; *N., Nocardia*.
[b] Kir^r, kirromycin resistant; Kir^s, kirromycin sensitive.

S. coelicolor tuf3 is subject to positive stringent control (36), and the *tuf3* gene product can function as a real EF-Tu in a *Streptomyces* in vitro translation system (24).

The lack of *tuf2* homologues in all *Streptomyces* species studied so far (35, 37; L. N. Olsthoorn-Tieleman, unpublished results) and the apparent absence of a *tuf2* gene product in *S. ramocissimus* (37) raised the question of whether *tuf2* encodes an EF-Tu with a general or specialized function. In this paper we provide the sequences of the flanking genes of *S. ramocissimus tuf2* and perform a transcriptional analysis of *tuf2* and describe the overexpression and purification of its gene product. The actual functioning of EF-Tu2 as an EF-Tu and its interaction with kirromycin were studied by using a recently developed *Streptomyces* in vitro translation system (24).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and vectors. Elfamycin-producing *Actinomyces* strains used are listed in Table 1. *E. coli* JM101 (26) and ET12567 (18), grown and transformed by standard procedures (26), were used for routine subcloning. All DNA manipulations were performed by following standard protocols given by Sambrook et al. (26). pUSRT2 was constructed by cloning the 2.9-kb *Bam*HI fragment from pASRT2 (37) in *Bam*HI-digested pUC18 (41). pUSRT2-U1 and pUSRT2-5 contain the 0.4-kb *Bam*HI/*Sac*II fragment of pUSRT2-1 (37) cloned into the *Bam*HI/*Sac*I sites of pUC18 and the 0.6-kb *Bam*HI/*Bcl*I fragment of pUSRT2-1 cloned in *Bam*HI-digested pUC18, respectively.

S. ramocissimus strains B7 and CBS 190.6 (wild-type), both obtained from Gist-brocades NV (Delft, The Netherlands), were grown as liquid cultures in S medium for the isolation of chromosomal DNA and EF-Tu1. SFM medium (containing, per liter, 20 g of mannitol, 20 g of soy flour, and 20 g of agar dissolved in tap water and autoclaved twice) is a modified version of that reported by Hobbs et al. (10) and was used to make high-titer spore suspensions of *S. ramocissimus* B7. Conditions for reproducibly dispersed growth of *S. ramocissimus* B7 in NMMP medium (11) containing 1% (wt/vol) glucose were as described by Tieleman et al. (32). Kirromycin response was induced in liquid cultures by adding kirromycin to a final concentration of 5 μM at an optical density at 450 nm (OD_{450}) of 0.6, after which the cultures were allowed to continue growing. *S. ramocissimus* B7 spores were plated on cellophane disks on AMMAT medium (32) to facilitate the harvesting of the mycelium for RNA isolation. Morphology of the surface-grown cultures was determined by phase-contrast microscopy, while kirromycin secretion into the agar was detected by using *E. coli* JM101 as the indicator strain.

S. coelicolor M145 (11) was obtained from the John Innes Centre, Norwich, United Kingdom; the construction of *S. coelicolor* J1501 derivative LT2 is described by Olsthoorn-Tieleman et al. (24). *S. coelicolor* strains were grown in YEME medium (11) and on R5 plates (11), when necessary supplemented with 1% (wt/vol) mannitol, 7.5 μg of uracil/ml, and 50 μg of histidine/ml, as described previously (11). MSP (2% [wt/vol] mannitol, 2% [wt/vol] soy peptone) was used to grow *S. coelicolor* LT2 for in vitro translation experiments. Protoplast preparation and transformation were performed as described by Hopwood et al. (11).

Southern hybridization. Chromosomal DNA from the different elfamycin-producing actinomycetes was isolated from liquid cultures grown in S medium according to the method described by Hopwood et al. (11) and digested with the appropriate enzymes. Southern blotting and hybridization were performed under conditions described previously (24). The 1.0-kb *Mlu*I/*Nco*I, 1.0-kb *Nar*I, and 0.65-kb *Sal*I fragments of *S. ramocissimus tuf1*, *tuf2*, and *tuf3*, respectively, were used as probes after ^{32}P labeling by random priming (7). The hybridization stringency was set at 6× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate, pH 7)–0.6% sodium dodecyl sulfate (SDS) at 65°C, and final washes were with 2× SSC–0.1% SDS at the same temperature.

DNA sequence analysis. The nucleotide sequence of the *tuf2* downstream region was determined by dideoxy sequencing using the Pharmacia T7 sequencing kit and single-stranded DNA templates derived by subcloning DNA fragments from pUSRT2 in M13mp18 and M13mp19 (41). Synthetic oligonucleotides were used to close gaps in the sequence. Sequence analyses were performed using the Wisconsin GCG package (6). BLAST search engines BlastN, BlastP, and BlastX (2) were used to perform database searches.

Promoter probing experiments. pISRT2*xylE*-1 and pISRT2*xylE*-1*i* were constructed by cloning the *Bam*HI/*Sac*II fragment of *tuf2*-containing plasmid pUSRT2-1 (37) via pUC18 into *xylE*-based promoter probe vector pIJ4083 (4a) in both orientations. Transformants containing either pISRT2xylE-1 or pISRT2xylE-1*i* were grown on R5 in the presence of 5 μg of thiostrepton (a gift from Squibb, Princeton, N.J.)/ml. Plates were sprayed with 0.5 M catechol after 2 to 5 days of growth, and the amount of catechol converted into yellow 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase was assessed visually.

Nuclease S1 protection assays. RNA was isolated from liquid- and surface-grown *S. ramocissimus* B7 cultures as described by Hopwood et al. (11), except that DNase I treatment was used in addition to salt precipitation to eliminate DNA from the nucleic acid preparations. RNA concentrations were determined spectrophotometrically, and the quality of the preparations was checked by gel electrophoresis. Hybridization of 30 μg of RNA with the appropriate DNA probes was performed in sodium trichloroacetate-based buffer (22) at 45°C overnight after denaturation at 70°C for 15 min. All subsequent steps were carried out as described previously (30) with an excess of probe. The 510-bp *Bsp*120I/*Pvu*II fragment from pUSRT2-5, ^{32}P end labeled at the 5′ end of the *Bsp*120I site, was used for mapping *tuf2* transcripts. The *tuf1* and *tuf3* genes have no homology with this probe, thus excluding the possibility that these mRNAs would contribute to the protection pattern. The 600-bp *Bam*HI/*Pvu*II fragment from pUSRT2-U1, ^{32}P end labeled at the 5′ end of the *Bam*HI site, was used for mapping *orfQ* transcripts. Products were analyzed on denaturing 6% polyacrylamide gels, using ^{32}P-end-labeled *Hpa*II fragments of pBR322 as size markers.

Construction of *tuf2* overexpression vector pISRT2-1. The *S. ramocissimus tuf2* gene was isolated from plasmid pUSRT2-3 (37) as a 1.5-kb *Bam*HI/*Hgi*AI fragment and ligated into the *Bam*HI/*Pst*I sites of vector pUC18 (41), thereby creating pUSRT2-4. The *tuf2* gene was isolated from this vector as a *Bam*HI-*Hin*dIII fragment and cloned into the corresponding restriction sites of pIJ4070 (a kind gift from M. J. Bibb, Norwich, United Kingdom), resulting in pUSRT2*ermE*. In this way the gene was placed under the control of the strong and constitutive *Streptomyces ermE* promoter. Finally pISRT2-1 was constructed by inserting the *tuf2* gene and upstream *ermE* promoter as a 1.7-kb *Kpn*I/*Pst*I fragment into the *Kpn*I/*Pst*I sites of *Streptomyces* high-copy-number vector pIJ487 (40).

Production and purification of *S. ramocissimus* EF-Tu species. Exponentially growing *S. ramocissimus* B7, cultured in S medium, was used as a source for

EF-Tu1. EF-Tu2 overproduction was achieved by inoculation of fresh spores from *S. coelicolor* LT2 harboring pISRT2-1 into YEME containing 5 μg of thiostrepton/ml and growth for 64 h at 30°C. The mycelium was washed twice with ice-cold Tu^{Gly} buffer (50 mM Tris-HCl [pH 7.6], 7 mM $MgCl_2$, 60 mM NH_4Cl, 1 mM dithiothreitol, 10 μM GDP, 10 μM phenylmethylsulfonyl fluoride, 10% [vol/vol] glycerol) and kept frozen at −80°C. For the purification of *S. ramocissimus* EF-Tu1 and EF-Tu2, the method described by Olsthoorn-Tieleman et al. (24) for *S. coelicolor* EF-Tu1 was used with the following modifications. Before applying the EF-Tu2-containing S100 fraction on the DEAE-Sepharose column, it was first subjected to chromatography on a Ni^{2+}-nitrilotriacetic acid (NTA) agarose column to remove *S. coelicolor* His_6-tagged EF-Tu1 (EF-Tu1His). In addition to the described purification protocol, the EF-Tu1 and EF-Tu2 protein solutions were applied to an fast protein liquid chromatography Mono Q column and eluted using a linear gradient of KCl (140 to 500 mM) in Tu^{Gly} buffer for further purification. The protein solutions were concentrated over Amicon Centriflo ultrafiltration cones and stored at −80°C. Protein concentrations were determined with Coomassie protein assay reagent (Pierce) by using bovine serum albumin as a standard.

SDS-PAGE and Western analysis. Protein expression and purification was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) using the Mini Protean II system (Bio-Rad) and Western blotting conducted as described by Vijgenboom et al. (37), using a 1:5,000 dilution of antibodies. Nonradioactive detection was performed using Western blot chemiluminescence reagent (NEN Life Science Products). The rabbit polyclonal antibodies used were raised against the *S. ramocissimus tuf3* gene product expressed in *E. coli* (anti-EF-Tu3) and against *S. ramocissimus* EF-Tu1 (anti-EF-Tu1) (37).

In vitro translation assays in the absence and presence of antibiotics. S30 cell extracts of *S. coelicolor* LT2 (if necessary harboring pISRT2-1 or pIJ487), from which His_6-tagged EF-Tu1 was removed by treatment with Ni^{2+}-NTA-agarose beads, were obtained as described by Olsthoorn-Tieleman et al. (24). The extracts, supplemented with purified EF-Tu species and antibiotics at various concentrations, were incubated in translation buffer (final concentrations: 50 mM Tris-HCl [pH 7.6], 9 mM $MgCl_2$, 60 mM NH_4Cl, 1 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 6 mM phosphoenolpyruvate, 50 μg of pyruvate kinase/ml, 0.1 mg of poly[U]/ml, 0.2 mg of *E. coli* tRNA/ml, and 13.2 μM [^{14}C]Phe [specific activity, 531 mCi/mmol]) at 30°C for 10 min. The total volume was 50 μl. The reaction was stopped by the addition of 15 μl of 1 M NaOH and further incubation at 30°C for 10 min. After precipitation with 5% (wt/vol) trichloroacetic acid and filtration, the incorporation of [^{14}C]Phe was determined by liquid scintillation counting.

Antibiotics kirromycin and pulvomycin were generous gifts from Gist-brocades NV and A. Parmeggiani (Palaiseau, France), respectively. GE2270 A was isolated by V. G. Möhrle (Leiden University, Leiden, The Netherlands) as described by Selva et al. (28).

Nucleotide sequence accession number. The sequence of the *tuf2* downstream region has been deposited in the GenBank under accession no. AY062294.

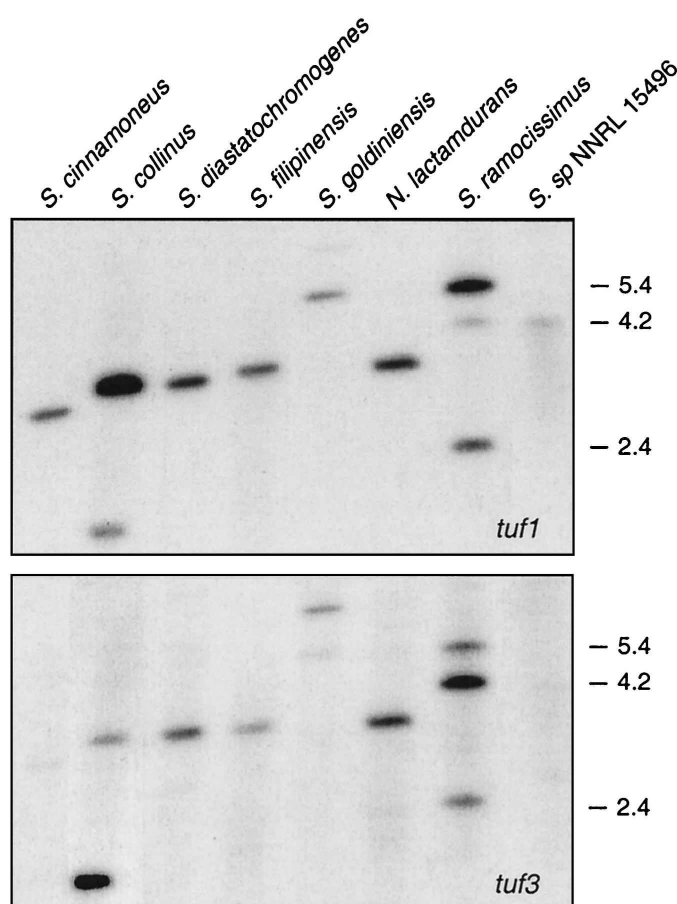

FIG. 1. Southern analysis of the number of *tuf* genes in elfamycin-producing actinomycetes. Chromosomal DNA of the producers of kirromycin-like antibiotics was digested with *Bam*HI and *Pst*I, except for *Streptomyces* sp. strain NNRL 15496 DNA, which was restricted with *Bgl*II and *Sph*I, and probed with internal sequences of *S. ramocissimus tuf1* (top) and *tuf3* (bottom). The positions and approximate sizes (in kilobases) of the *S. ramocissimus* fragments that hybridize with the probes are indicated.

RESULTS

Number and character of *tuf* genes in elfamycin-producing actinomycetes. The presence of three *tuf* genes in *S. ramocissimus*, in contrast to the two *tuf1* and *tuf3* homologues in several other streptomycetes (35, 37; L. N. Olsthoorn-Tieleman, unpublished results), raised the question of whether the occurrence of *tuf2* is a particular feature of producers of kirromycin-type antibiotics. Therefore, Southern hybridizations employing different digests of chromosomal DNA from several elfamycin producers were carried out using internal fragments of the three *S. ramocissimus tuf* genes as probes. Hybridization with the *tuf1* probe revealed three hybridizing fragments in *Bam*HI- and *Pst*I-digested *S. ramocissimus* DNA (Fig. 1, top). As described by Vijgenboom et al. (37), the two strong signals of 5.4 and 2.4 kb correspond to *tuf1* and *tuf2*, respectively, while the weaker signal of 4.2 kb indicates the presence of *tuf3*. In the restriction digests of the other actinomycetes only one strong signal was observed; in some lanes an additional weaker signal was also present. An identical hybridization pattern was observed when the *tuf2* probe was used (data not shown), indicating that all actinomycetes investigated possess a *tuf1* homologue and that only *S. ramocissimus* contains the additional *tuf2* gene. Hybridization with the internal fragment of *S. ramocissimus tuf3* (Fig. 1, bottom) revealed that *Streptomyces collinus* and *Streptomyces goldiniensis* contain a close homologue of this divergent *tuf* gene. The *tuf1*- and *tuf3*-hybridizing fragments of *Streptomyces filipinensis* seemed to have migrated to slightly different positions, and inspection of *Bgl*II- and *Sph*I-digested DNA confirmed the presence of two distinct *tuf* genes (data not shown). The *Bam*HI- and *Pst*I-restricted genomic DNAs of *Streptomyces fradiae* (not shown in Fig. 1) and *S. goldiniensis* exhibited the same hybridization patterns with both probes, indicating that the former strain also contains *tuf1* and *tuf3* homologues. The established number and character of *tuf* genes in each elfamycin-producing strain were confirmed by similar Southern analyses using other restriction digests.

Summarizing, the presence of *tuf2* seems to be restricted to *S. ramnocissimus* B7 (Table 1), suggesting a specialized function of the gene for this specific microorganism. Since B7 is a

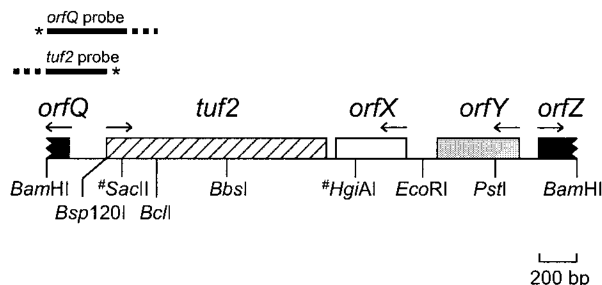

FIG. 2. Organization of the *S. ramocissimus tuf2* region. A restriction map of the insert from pUSRT2 is shown. Only the *Sac*II and *Hgi*AI restriction sites relevant to the text are shown (#). The probes used for S1 nuclease mapping of *tuf2* and *orfQ* transcripts (asterisks, ^{32}P-labeled 5′ ends) are shown above the restriction map. Dashed lines, nonhomologous pUC18-derived extensions.

mutant strain that was isolated during selection for increased kirromycin production, the number of *tuf* genes in the parental strain, CBS 190.6, was also determined by Southern blot experiments (data not shown). Exactly the same hybridization pattern was observed, indicating that the presence of three *tuf* genes in *S. ramocissimus* B7 is not an artifact due to the screening procedure.

The genes flanking *S. ramocissimus tuf2* are not involved in protein biosynthesis. In most bacteria the genes for EF-Tu are found in operons together with genes encoding other components of the translational apparatus, such as ribosomal proteins, EF-G, and tRNAs (17). Close inspection of the DNA sequence preceding *S. ramocissimus tuf2* as published by Vijgenboom et al. (37) (GenBank accession no. X67058) revealed the presence of an incomplete open reading frame (ORF) with protein-coding character, designated *orfQ*, in the orientation opposite to that of *tuf2* (Fig. 2). Comparison of the putative gene product with proteins in the databases revealed significant homology (60% amino acid identity in an overlap of 40 amino acids [aa]) to the N-terminal part of a putatively secreted protein of *S. coelicolor* (GenBank accession no. CAC14349).

To further study the genetic organization of *S. ramocissimus tuf2*, the sequence of a 1.3-kb region downstream of *tuf2* was determined (GenBank accession no. AY062294; nucleotide [nt] 1 corresponds to nt 1500 of X67058). This revealed two complete ORFs with protein-coding character, as well as the beginning of a third (Fig. 2). The first ORF (*orfX*; nt 448 to 65) is located 48 nt downstream of and in the opposite orientation to *tuf2*. *orfX* encodes a protein of 127 aa with a calculated molecular mass of 13.4 kDa; this protein shows a significant degree of amino acid sequence identity to anti-sigma factor antagonists, the most similar one being from *S. coelicolor* (GenBank accession no. CAC14346; 67% amino acid sequence identity in an overlap of 116 aa). Intriguingly, the last codon of *orfX* is the rare TTA leucine codon, potentially subject to *bldA*-dependent translational control (16). The second ORF, designated *orfY*, spans from nt 1054 to 617 and is also oriented in the opposite direction to *tuf2*. Its predicted product of 145 aa (16.3 kDa) shows homology to transcription regulators of the MarR family such as one from *S. coelicolor* (GenBank accession no. CAC17527), with 32% amino acid sequence identity in an overlap of 125 aa. The third downstream ORF of >226 nt, *orfZ*, starts at nt 1159 and runs into the same direction as *tuf2*; its 3′ end appears to be outside of the sequenced region. Searches of the databases revealed the high similarity of the product to aldehyde dehydrogenases (50% amino acid sequence identity in an overlap of 68 aa with *Pseudonocardia* sp. strain K1 succinate semialdehyde dehydrogenase; GenBank accession no. CAC10505).

These data reveal that in contrast to the operon organization of other *tuf* genes, no genes for components of the translational apparatus are found in the immediate vicinity of *tuf2*. The opposite orientations of the flanking genes *orfQ* and *orfX* with respect to *tuf2* suggest that *tuf2* is located in a single transcription unit.

A bidirectional promoter region precedes *S. ramocissimus tuf2*. The absence of detectable *tuf2* expression in total protein extracts of *S. ramocissimus* (37) suggests that *tuf2* is expressed at very low levels under normal growth conditions or that *tuf2* might even be a silent gene. To distinguish between these possibilities, we decided to determine if the *tuf2* upstream region displays promoter activity and if *tuf2* transcripts could be observed with the sensitive S1 nuclease mapping technique.

To determine the presence and approximate location of the promoter(s) in the *tuf2-orfQ* intergenic region, we used multicopy promoter-probe vector pIJ4083 (4a), which contains the promoterless *xylE* as the reporter gene. The *tuf2* upstream region was cloned as a 410-bp *Bam*HI/*Sac*II fragment in both orientations in pIJ4083, with the start of *tuf2* (pISRT2*xylE*-1) or of *orfQ* (pISRT2*xylE*-1*i*) proximal to *xylE*. *S. coelicolor* M145 transformants containing either pISRT2xylE-1 or pISRT2xylE-1*i* yielded yellow aerial hyphae when sprayed with catechol after at least 3 days of growth on R5 agar plates, indicative of the presence of at least two divergent promoters in the *Bam*HI/*Sac*II fragment. Control transformants harboring pIJ4083 without an insert displayed no yellow coloring upon being sprayed with catechol.

To map the possible transcription start sites of both *tuf2* and *orfQ*, nuclease S1 protection experiments were carried out with RNA isolated from exponentially growing *S. ramocissimus* (for details see reference 32). The 510-bp *Bsp*120I/*Pvu*II fragment from pUSRT2-5 and the 600-bp *Bam*HI/*Pvu*II fragment from pUSRT2-U1, uniquely labeled at their 5′ ends, were used as probes to detect *tuf2* and *orfQ* transcripts, respectively; in both cases the nonhomologous pUC18-derived extension allowed discrimination between full-length RNA-protected fragments and the reannealed probe. The *tuf2* probe contains no *tuf2* coding sequence, because unwanted detection of the abundantly present and almost identical *tuf1* transcripts would then likely occur. Only after prolonged exposure of the autoradiograms were *tuf2* and *orfQ* transcripts detected (*tuf2* data in Fig. 3, right, lane C; *orfQ* data not shown). The putative transcriptional initiation sites for *tuf2* and *orfQ* were identified around nt 20 and 290 (*tuf2*p1 and *orfQ*p1, respectively). Inspection of the DNA sequences preceding these start sites revealed no similarity to the proposed consensus sequence 5′TTGACR-16 to 18 nt-TAGRRT3′, where R is G or A, for promoters recognized by the major RNA polymerase holoenzyme of *Streptomyces* (31).

Growth phase-dependent transcription of *tuf2* in surface-grown cultures producing kirromycin. Surface-grown cultures of streptomycetes show complete morphological differentia-

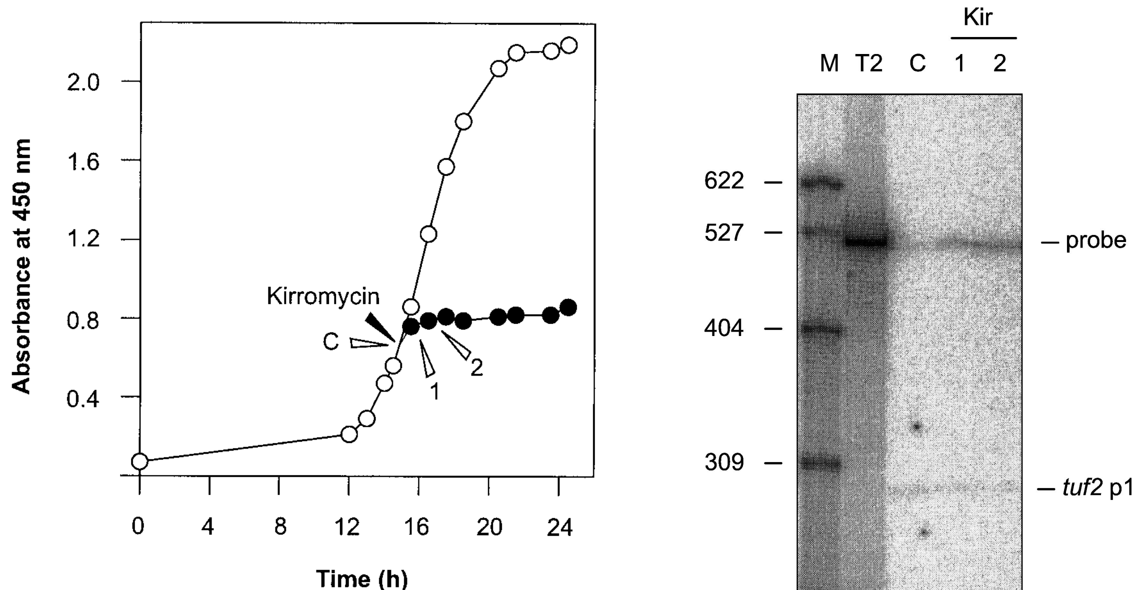

FIG. 3. Transcription of *tuf2* after kirromycin addition. (Left) Growth curve of *S. ramocissimus* B7 with (●) and without (○) the addition of kirromycin (5 μM) at an OD_{450} of 0.6. Addition of the antibiotic to a final concentration of 25 μM resulted in a similar growth inhibition. (Right) S1 nuclease protection analysis of *tuf2* transcripts in RNA isolated at the time points indicated in the left panel (C, 1, and 2). Probe, reannealed *tuf2* probe; *tuf2*p1, transcripts initiated at *tuf2*p1. In lane T2, the location of the 510-nt full-length *tuf2* probe can be seen. Lane M, end-labeled *Hpa*II-digested pBR322 size markers (sizes are given in nucleotides).

tion. To study *tuf2* transcription during the life cycle of *S. ramocissimus* B7 and to assess the relationship between *tuf2* transcription and kirromycin production, S1 nuclease protection experiments were performed with RNA isolated from surface-grown cultures at different stages of growth by using the *tuf2* probe as described above. Barely detectable levels of *tuf2* transcripts were observed during the formation of vegetative hyphae, with transcription initiating from the same start site (*tuf2*p1) as found for the liquid culture (data not shown). No transcripts were present during aerial mycelium development and kirromycin production, but *tuf2* transcripts reappeared during the sporulation stage. Thus, *tuf2* transcription from *tuf2*p1 shows the same growth phase dependence as *S. ramocissimus tuf1* (32), albeit at a much lower level.

Since a role for an EF-Tu-like protein in the kirromycin resistance mechanism of *S. ramocissimus* is imaginable, we analyzed how *tuf2* transcription responded to the addition of kirromycin. The antibiotic (final concentration, 5 μM) was added to exponentially growing *S. ramocissimus* B7 liquid cultures at an OD_{450} of 0.6, after which the cultures continued growing (albeit at a slow rate) and stopped growing about 2 h later (Fig. 3, left). S1 nuclease protection experiments with RNA isolated from these cultures 1 and 2 h after addition of the inhibitor revealed that the level of *tuf2* transcripts remained unaltered (Fig. 3, right). Thus, *tuf2* transcription from *tuf2*p1 is unaffected by externally added kirromycin, ruling out the possibility that the *tuf2* gene product might be directly involved in conferring resistance to kirromycin.

***S. ramocissimus tuf2* encodes a functional EF-Tu.** Expression of the *tuf2* gene could not be demonstrated in total-protein extracts of *S. ramocissimus*, and heterologous expression of *tuf2* in *E. coli* yielded a small amount of inactive product, deposited in inclusion bodies (37). To achieve high expression of active EF-Tu2, *tuf2* was cloned behind the constitutive *ermE* promoter in pIJ487, resulting in *Streptomyces* expression vector pISRT2-1 (for details see Materials and Methods). Since a suitable transformation system for *S. ramocissimus* was not found, overexpression of *tuf2* in *S. coelicolor* M145, a strain naturally lacking a *tuf2* analogue, was studied (35). Constitutive overexpression of the plasmid-borne *tuf2* gene affected the host cell, resulting in growth retardation and aberrant production of red pigments. S30 extracts prepared from cultures of pISRT2-1 transformants grown for 40 h in YEME were analyzed by Western blotting. As shown in Fig. 4, high expression of *tuf2* was achieved; the overexpressed product was also clearly visible in Coomassie brilliant blue-stained gels (data not shown). Despite its slightly higher calculated molecular mass (44.1 versus 43.6 kDa), EF-Tu2 migrates considerably faster than *S. coelicolor* EF-Tu1 during SDS-PAGE, consistent with the results of heterologous expression in *E. coli* (37). Two types of polyclonal antibodies, anti-EF-Tu1 and anti-EF-Tu3, raised against the *S. ramocissimus tuf1* and *tuf3* gene products, respectively, showed high cross-reactivities to EF-Tu2. Additional minor bands at much higher or lower positions represent nonspecific interactions with S30 extract proteins.

The homology between *S. ramocissimus* EF-Tu2 and EF-Tu1 (88% amino acid sequence identity) and the conservation of the proposed binding sites for both aminoacyl-tRNA (23) and EF-Ts (13) suggest that EF-Tu2 could also sustain trans-

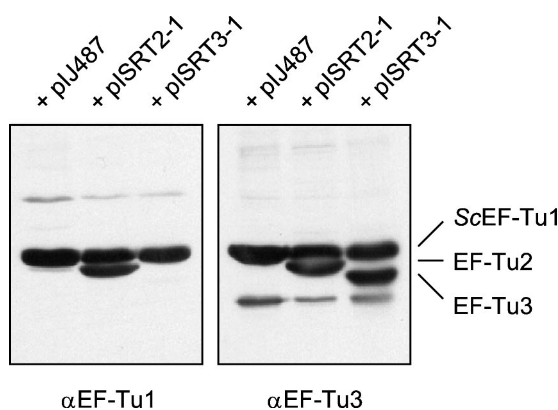

FIG. 4. Overexpression of the *S. ramocissimus tuf2* and *tuf3* gene products in *S. coelicolor* M145. S30 extracts of *S. coelicolor* M145 transformed with different overexpression plasmids were analyzed by Western blotting using polyclonal antibodies raised against EF-Tu1 (left) or EF-Tu3 (right). Left lane, *S. coelicolor*(pIJ487); middle lane, *S. coelicolor*(pISRT2-1); right lane, *S. coelicolor*(pISRT3-1).

lation in *S. ramocissimus*. To explore the possible participation of this protein in the elongation cycle, in vitro translation experiments were performed with complete cell S30 extracts from *S. coelicolor* LT2 harboring EF-Tu2 expression vector pISRT2-1. *S. coelicolor* LT2 is a J1501 derivative modified in both *tuf* genes: the *tuf3* gene is disrupted and *tuf1* is replaced by *tuf1His*, encoding EF-Tu1His as the only functional EF-Tu (24). As shown in Fig. 5a, translational activity was observed in an extract from LT2 harboring pISRT2-1, even after removal of EF-Tu1His by Ni^{2+} affinity adsorption, indicating that the overexpressed EF-Tu2 is responsible for protein synthesis in vitro. A control experiment was performed with a Ni^{2+}-NTA-treated extract of LT2 containing pIJ487 as the parental vector without *tuf2*, and virtually no activity was measured. The predicted presence or absence of the different EF-Tu species in the S30 extracts was confirmed by Western blotting (Fig. 5a).

To investigate whether EF-Tu2 functions as well as EF-Tu1 in poly(U)-directed poly(Phe) synthesis, the protein was purified from *S. coelicolor* LT2 harboring pISRT2-1 as described in Materials and Methods. The factors were used to complement a *Streptomyces* EF-Tu-dependent in vitro translation system (24), and their translational capacities as a function of the EF-Tu concentration were studied. As can be concluded from Fig. 5b, the extents of [^{14}C]Phe incorporation in vitro for the two proteins were comparable.

***S. ramocissimus* EF-Tu2 is sensitive to kirromycin.** The preceding results indicate that *S. ramocissimus* EF-Tu2 is perfectly able to sustain the whole elongation process in vitro. This prompted us to investigate whether EF-Tu2 is able to promote translation in the presence of kirromycin and thus is somehow involved in the kirromycin resistance mechanism of *S. ramocissimus*. Resistance to kirromycin is usually achieved by single amino acid substitutions in highly conserved positions of EF-Tu. These mutations cluster in or near the interface between domains I and III of EF-Tu·GTP (1, 19), a region where the antibiotic was recently found to bind (38). Superposition of the EF-Tu2 amino acid sequence on the crystal structure of *Thermus thermophilus* EF-Tu·GppNHp (3) revealed that no deviating residues are present at the interface between domains I and III, although kirromycin resistance due to residues elsewhere in the protein cannot be excluded.

Therefore the kirromycin sensitivities of both *S. ramocissimus* EF-Tu2 and EF-Tu1 in the *Streptomyces* in vitro translation system were monitored. As can be concluded from Fig. 6a, EF-Tu2 appeared to be as sensitive to kirromycin as EF-Tu1, with a concentration at which 50% inhibition of poly[U]-directed poly[Phe] synthesis is observed of 0.05 μM. Also no difference in sensitivity to two other EF-Tu-targeted antibiot-

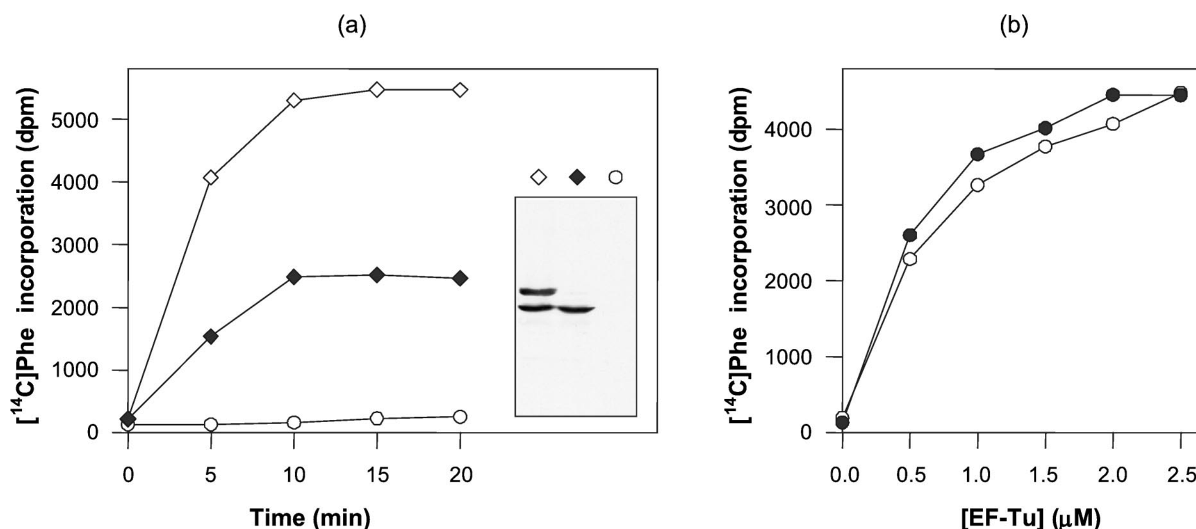

FIG. 5. Translational activity of *S. ramocissimus* EF-Tu2. (a) Translational activity of cell extracts of *S. coelicolor* LT2 harboring expression vector pISRT2-1 with (◇) and after removal of (◆) endogenous EF-Tu1His. A Ni^{2+}-NTA-treated cell extract of *S. coelicolor* LT2 harboring pIJ487, the parental vector without *tuf2*, was used as a control (○). (Inset) Corresponding Western blot of the three extracts. (b) In vitro translation of an EF-Tu-depleted *S. coelicolor* cell extract supplemented with *S. ramocissimus* EF-Tu2 (●) and *S. ramocissimus* EF-Tu1 (○). The translation of the poly(U) messenger was studied by measuring the incorporation of [^{14}C]Phe at 30°C as a function of time (a) and of the concentrations of the EF-Tu species during a 10-min incubation (b).

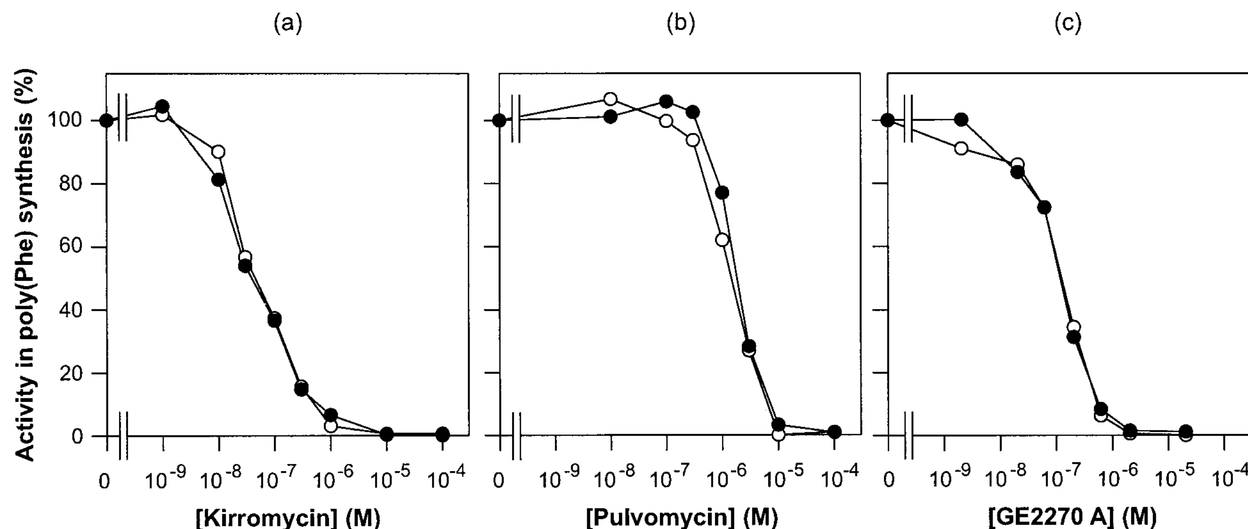

FIG. 6. Translational activities of EF-Tu1 and EF-Tu2 in the presence of antibiotics. Shown is in vitro translation of an EF-Tu-depleted *S. coelicolor* cell extract supplemented with 1.0 μM *S. ramocissimus* EF-Tu1 (○) or *S. ramocissimus* EF-Tu2 (●). The translation of the poly(U) messenger was studied by measuring the incorporation of [^{14}C]Phe at 30°C as a function of the concentrations of kirromycin (a), pulvomycin (b), and GE2270 A (c). For both EF-Tu1 and EF-Tu2 the activity in the absence of the antibiotics was normalized to 100% (the two EF-Tu preparations were about equally active, as shown in Fig. 5b).

ics, pulvomycin and GE2270 A, between the two EF-Tu proteins was observed, with 50% inhibition occurring at about 1.5 and 0.1 μM, respectively (Fig. 6b and c). Complex formation of EF-Tu2·GDP with these three inhibitors, studied by electrophoresis under nondenaturing conditions, revealed that band shifts for EF-Tu1 were similar to those for EF-Tu2 (data not shown), confirming that the binding sites on EF-Tu2 for these three antibiotics are intact.

These data demonstrate that the minor EF-Tu2 and the major EF-Tu1 of kirromycin producer *S. ramocissimus* are indistinguishable in their abilities to promote poly(Phe) synthesis in vitro and in their sensitivities to kirromycin and other EF-Tu-targeted antibiotics.

DISCUSSION

Unlike the situation in higher eukaryotes, the occurrence of gene families is not a prominent feature of bacteria, although the presence of duplicate genes encoding EF-Tu (*tufA* and *tufB*) in several gram-negative bacteria has been demonstrated (8, 27). Their locations, one *tuf* gene linked to certain ribosomal protein genes and the other linked to tRNA genes, are also conserved among distantly related bacterial species (14). The maintenance of two active *tuf* copies, contributing about equally to the total EF-Tu concentration, has been ascribed to evolutionary pressure for an increased amount of protein product and an increased flexibility in expression regulation. The presence of three heterogeneous *tuf* genes in kirromycin producer *S. ramocissimus* is intriguing. While streptomycetes generally contain *tuf1* and *tuf3* homologues, *tuf2* has no equal in the actinomycetes studied so far (4, 21, 35, 37; L. N. Olsthoorn-Tieleman, unpublished results). Here we have demonstrated the absence of *tuf2* in several producers of kirromycin-type antibiotics. Furthermore, we have gathered information about this unique *tuf2* gene and its gene product, EF-Tu2, to determine the role of this additional EF-Tu-like protein in *S. ramocissimus*.

Unlike other *tuf* genes, *tuf2* is not linked to other genes for components of the translational apparatus but rather seems to be located in a single transcription unit. Low-level transcription from a promoter within the oppositely transcribed *orfQ* takes place, as demonstrated by promoter-probing experiments and S1 nuclease protection assays. Transcription of *tuf2* shows a growth phase dependence similar to that of *S. ramocissimus tuf1* (32), although *tuf1* and *tuf2* transcript levels differ by at least several orders of magnitude during normal growth in either liquid cultures or on agar plates. As a result, EF-Tu2 does not contribute significantly to the total EF-Tu pool in *S. ramocissimus.* The lack of *tuf2* transcripts during kirromycin production and the unresponsiveness of *tuf2* transcription to kirromycin induction argue against a role for *tuf2* as a kirromycin resistance determinant.

Purified EF-Tu2 was perfectly able to sustain poly(Phe) synthesis in a *Streptomyces* in vitro translation system and was indistinguishable in this ability from the regular EF-Tu1. Its measured sensitivity to kirromycin eliminates any possibility that this additional elongation factor might be directly involved in conferring resistance to kirromycin. The degree of similarity between EF-Tu1 and EF-Tu2 (88% amino acid sequence identity) is consistent with the notion that the proteins are functionally homologous in vitro but also implies that certain structural and functional differences might exist. It should be noted that *S. ramocissimus* EF-Tu1 is much more similar (96% amino acid sequence identity) to the EF-Tu1 proteins from *S. coelicolor* (35), *S. collinus* (20), and *Streptomyces netropsis* (L. N. Olsthoorn-Tieleman, unpublished results). Comparison of both *S. ramocissimus* EF-Tu protein sequences with a defined common eubacterial EF-Tu sequence (24) revealed that EF-Tu2 contains six deviations (I199V, D/E240T, L/I292V,

K294R, V/I308A, and K/E390R), while EF-Tu1 differs in only one position (V/I308A) (*E. coli* EF-Tu numbering is used throughout). Residues 240, 292, and 294 are in an area of domain II that has been implicated in the interaction with the ribosome (29, 34, 39). Answers about the role of *tuf2* in vivo might be found by succeeding in *tuf* gene disruption experiments with *S. ramocissimus*, which are currently hampered by the presence of an efficient restriction modification system (L. N. Olsthoorn-Tieleman, unpublished results).

The main points to keep in mind for determination of the function of EF-Tu2 are (i) the unique presence of *tuf2* in kirromycin producer *S. ramocissimus*, excluding a general role in *Streptomyces* spp., (ii) the different genetic environment of *tuf2* compared to those of other *tuf* genes, hinting at some primary function other than acting as a translational EF, and (iii) the extremely low *tuf2* expression level in comparison with that of *tuf1*, ruling out the possibility that EF-Tu2, although capable of sustaining poly(Phe) synthesis, plays a significant role in normal protein biosynthesis. For now, we tentatively conclude that *S. ramocissimus* EF-Tu2 plays a regulatory role, which requires only trace amounts of protein, rather than having a function as an additional EF-Tu.

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