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Three *tuf*-like genes in the kirromycin producer *Streptomyces ramocissimus*

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We have identified, cloned and sequenced three *tuf*-like genes from *Streptomyces ramocissimus* (Sr.), the producer of the antibiotic kirromycin which inhibits protein synthesis by binding the polypeptide chain elongation factor EF-Tu. The *tuf-1* gene encodes a protein with 71% amino acid residues identical to the well characterized elongation factor Tu of *Escherichia coli* (Ec.EF-Tu). The genetic location of *tuf-1* downstream of a *fus* homologue and the *in vitro* activity of Sr.EF-Tu1 show that *tuf-1* encodes a genuine EF-Tu. The putative Sr.EF-Tu2 and Sr.EF-Tu3 proteins are 69% and 63% identical to Ec.EF-Tu. Homologues of *tuf-1* and *tuf-3* were detected in all five *Streptomyces* strains investigated, but *tuf-2* was found in *S. ramocissimus* only. The three *tuf* genes were expressed in *E. coli* and used to produce polyclonal antibodies. Western blot analysis showed that Sr.EF-Tu1 was present at all times under kirromycin production conditions in submerged and surface-grown cultures of *S. ramocissimus* and in germinating spores. The expression of *tuf-2* and *tuf-3* was, however, below the detection level. Surprisingly, Sr.EF-Tu1 was kirromycin sensitive, which excludes the possibility that EF-Tu is involved in the kirromycin resistance of *S. ramocissimus*.

Keywords: *Streptomyces ramocissimus*, *tuf* genes, EF-Tu, kirromycin

INTRODUCTION

The polypeptide chain elongation factor EF-Tu of *E. coli* is one of the most abundant proteins in the bacterial cell and fulfils an essential function in protein synthesis. The factor is encoded by two so-called *tuf* genes (Jaskunas *et al.*, 1975; Yokota *et al.*, 1980; An & Friesen, 1980), which are well separated on the chromosome and are located in completely different genetic contexts (Lindahl *et al.*, 1977; Lee *et al.*, 1981). The gene products differ in one single amino acid at the C-terminus. The functional significance of this *tuf* gene 'duplication' is not well understood. The two genes in *E. coli*, *tufA* and *tufB*, are coordinately expressed in an 1:1 ratio throughout growth, indicating that the organism sets value on the presence of both genes (van der Meide *et al.*, 1983a, b).

The initial findings in *E. coli* led to studies of the *tuf* gene organization in various eubacteria (Sela *et al.*, 1989; Filer & Furano, 1981; Ludwig *et al.*, 1990) and an archaeobacterium (Lechner & Böck, 1987). Southern blot analysis revealed that the gene 'duplication' is widespread among Gram-negative bacteria (Filer & Furano, 1981). By contrast, the two Gram-positive bacteria, *Bacillus subtilis* and *Micrococcus luteus*, studied by these authors have only one *tuf* gene. These and later experiments led to the proposal that the difference in *tuf* gene number is characteristic for these two groups of bacteria. Similar studies, performed more recently and covering representatives of various phyla within the eubacterial kingdom, showed that there are exceptions to the rule. The fastidious Gram-negative bacterium *Chlamydia trachomatis* contains only one *tuf* gene (Goldstein *et al.*, 1989), as is also true for the extreme thermophilic bacterium *Thermotoga maritima* (Bachleitner *et al.*, 1989). One may even argue that *E. coli* does not meet the rule, since it harbours the *selB* gene encoding an additional EF-Tu, albeit a highly specialized one of strongly deviating size (Forchhammer *et al.*, 1989). Exceptions are also found in the group of Gram-positive eubacteria. Several strains of the

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genus *Clostridium* were shown to contain more than one *tuf* gene (Sela *et al.*, 1989).

The antibiotic kirromycin inhibits protein synthesis by binding to EF-Tu which causes retention of the factor on the ribosome and immobilization of the latter on the mRNA (Wolf *et al.*, 1977). Kirromycin belongs to the group of elfamycins, antibiotics with polyene structures (Edwards *et al.*, 1992) which are produced by the Gram-positive streptomycetes *S. ramocissimus*, *S. collinus* and a few others (Parmeggiani & Swart, 1985; Cundliffe, 1989). Streptomycetes are representatives of the morphologically more complex genera of the Gram-positive bacteria. The life-cycle of the streptomycetes starts with germinated spores forming branched vegetative mycelia. From these mycelia aerial hyphae grow erect, the ends of which are converted into chains of spores. When mature, these spores are released in a dormant state, whereafter germination reinitiates the whole process (Chater, 1989). During the transition phase, leading to the appearance of aerial hyphae, streptomycetes produce secondary metabolites such as a wide variety of antibiotics.

Various mechanisms are exploited by antibiotic-producing organisms to protect themselves against their own product (Cundliffe, 1989). The mechanism used by kirromycin producers, like *S. ramocissimus*, is essentially unknown (Glöckner & Wolf, 1984). Here we investigate the possibility that protection is achieved through the expression of a kirromycin-resistant EF-Tu species. Analysis of the *tuf* genes and their expression is a prerequisite. Much to our surprise we identified three rather divergent *tuf*-like genes, an unprecedented finding in prokaryotes. As a primary step towards identification of the products of the three *tuf*-like genes, they were heterologously expressed in *E. coli* using the *lac* promoter. With antibodies raised against two of the *tuf* gene products we studied the temporal expression of the three *tuf* genes in *S. ramocissimus*. In both liquid cultures during kirromycin production and in surface-grown cultures when complete development takes place, only *tuf-1* is abundantly expressed but *tuf-2* and *tuf-3* expression is below the detection level.

METHODS

Bacterial strains and plasmids. Cloning was done in *E. coli* strains 490, JM101 or JM109 (Messing, 1979; Yanisch-Perron *et al.*, 1985), using the plasmid pUC8 (Vieira & Messing, 1982) for *tuf-1*, and pAT153 (Twigg & Sherratt, 1980) for *tuf-2* and *tuf-3*. Subclones were made in pUC18 or pUC19 (Yanisch-Perron *et al.*, 1985). In the heterologous expression studies strain KA797 [*ara* Δ (*lac-proAB*) *thi* F' (*proAB*⁺ *lacI*^q), collection of the Department of Molecular Genetics, Leiden University, The Netherlands] was sometimes used instead of JM101. *S. ramocissimus* was obtained from Gist-brocades NV, The Netherlands.

Cloning strategy. Both *tuf-1* and *tuf-2* were isolated by a combination of Southern hybridization and a sib-selection procedure. For cloning of *tuf-1*, chromosomal DNA of *S. ramocissimus* was digested with *Bgl*II and ligated in *Bam*HI-digested pUC8. After transformation to *E. coli*, plasmid DNA was isolated from transformant pools, each pool representing

about 300 colonies. This DNA was screened by Southern hybridization with a *Hpa*I-*Nru*I fragment of the *E. coli tufA* gene as probe. The positive pools were subsequently reduced in size until the clone harbouring *tuf-1* (pUSRT1) was isolated. In order to identify the position of *tuf-1* on the *Bgl*II insert of pUSRT1, DNaseI deletion derivatives were generated essentially as described by Hong (1982). The location of *tuf-1* could be derived from the length of the insert, the *Sa*II restriction pattern and Southern analysis which showed whether the *Sa*II fragments that contain *tuf* sequences were still present. By this approach *tuf-1* was localized in the middle of the 2.8 kb *Bgl*II fragment.

For cloning of *tuf-2*, *Bam*HI-digested chromosomal DNA was fractionated on a 1% agarose gel and fragments with a length corresponding to the signal in a Southern blot probed with the *tuf-1* internal 330 bp and 240 bp *Sma*I fragments were ligated in pAT153. Clones were screened again by hybridization, using the sib-selection procedure. *tuf-2* was mapped on the positive clone (pASRT2) by digestion with various enzymes, such as *Sa*II, *Sma*I and *Pst*I, and identification of the fragments carrying *tuf*-like sequences by Southern hybridization. The *tuf-2* gene was thus localized at one end of the 2.8 kb *Bam*HI fragment.

Cloning of *tuf-3* was achieved in a one-step screening. The gene was localized on a 12 kb *Bam*HI fragment and a 9 kb *Bam*HI-*Eco*RI fragment by Southern hybridization using a 330 bp internal fragment from the *tuf-1* gene. *Bam*HI fragments with a length of 12 kb were isolated from an 1% agarose gel, digested with *Eco*RI and ligated in *Eco*RI- and *Bam*HI-digested pAT153. Since *Eco*RI sites are very rare in *Streptomyces* DNA this approach should yield a high percentage of positive clones. DNA isolated from *E. coli* transformants was analysed directly by Southern hybridization. The *tuf-3* gene was localized on the 9 kb *Bam*HI-*Eco*RI fragment (pASRT3) as described for *tuf-2*.

Southern hybridization. Chromosomal DNA was isolated as described by Hopwood *et al.* (1988). DNA from 1% agarose gels was blotted to Hybond-N. Filters were hybridized with ³²P-labelled probes in 6 × SSC, 0.1% SDS, 1 mM EDTA, 2 × Denhardt's solution [0.04% Ficoll (*M_r* 400 000), 0.04% BSA, 0.04% polyvinyl pyrrolidone (*M_r* 360 000)], 0.1% pyrophosphate, 200 μg calf thymus DNA ml⁻¹ for 16 h at 65 °C. When 50% formamide was used in the hybridization buffer, the incubation was done at 42 °C. Filters were washed in 6 × SSC at 65 °C and 2 × SSC at 65 °C for heterologous hybridizations and 0.5 × SSC or 0.2 × SSC at 65 °C for homologous hybridizations.

Dephosphorylated DNA probes were labelled with [³²P]ATP and T4 polynucleotide kinase, essentially as described by Maniatis *et al.* (1982), or labelled with the random prime method (Feinberg & Vogelstein, 1983, 1984).

DNA sequencing. Sequencing was done by the dideoxynucleotide method of Sanger *et al.* (1977) and M13mp18 or mp19 as vector (Yanisch-Perron *et al.*, 1985). 7-Deaza-2'-deoxyguanosine triphosphate was used to minimize band compression due to GC-rich regions

Growth of cultures. The *E. coli* strains were freshly transformed with the indicated plasmids and cultured in LB medium at 37 °C. Media were supplemented with 100 μg ampicillin ml⁻¹ and transcription from the *lac* promoter was induced by adding 0.5 mM IPTG. *Streptomyces* strains were cultured at 30 °C in baffled flasks in various liquid media: S medium (4 g peptone, 4 g yeast extract, 10 g glucose, 0.5 g MgSO₄·7H₂O, 2 g KH₂PO₄ and 2 g K₂HPO₄ per litre of water), TSB+1% Casamino acids, YEME or YMG (for details on media see Hopwood *et al.*, 1985). Solid media for surface-grown material

were: R2YE and MM (Hopwood *et al.*, 1985). For *S. ramocissimus* 200 ml of minimal medium was supplemented with 2 ml trace elements, 8 ml 250 mM CaCl₂, 3 ml 7.5 mg methionine ml⁻¹, 3 ml 7.5 mg leucine ml⁻¹ and 5 ml 15 mg tyrosine ml⁻¹. Mannitol (0.5%) was used as carbon source. Strains were grown on cellophane discs to facilitate harvesting of the mycelium.

Protein extraction. Cells were harvested by centrifugation. Thereafter all procedures were carried out at 4 °C. The cells were washed once in standard buffer (10 mM Tris/HCl pH 7.8, 60 mM NH₄Cl, 10 mM magnesium acetate, 0.05% β-mercaptoethanol, 1 mM PMSF). Cells were sonicated at 30 W on ice with 10 bursts of 45 s, allowing 15 s in between for cooling. The sonicated suspension was centrifuged at 30000 g for 30 min, yielding the S30 (supernatant) fraction. In some cases an additional centrifugation was performed at 100000 g for 2 h yielding the S100 (supernatant) fraction.

Isolation and purification of elongation factors. Electrophoretically homogeneous EF-Tu.GDP from *E. coli* and EF-Tu1.GDP from *S. ramocissimus* were isolated by affinity chromatography of S100 supernatant fractions over GDP-AH-Sepharose as described by Jacobson & Rosenbusch (1977) and by van der Meide *et al.* (1980). This procedure includes dialysis overnight of a mixture of the elongation factor and GDP-AH-Sepharose against buffer lacking GDP. The factor, bound to the AH-Sepharose, is eluted with a GDP-containing buffer.

The product of *tuf-1*, heterologously expressed in *E. coli*, was purified in two steps. First the S30 extract was put on a column of GDP-AH-Sepharose, which retains the *E. coli* EF-Tu but lets EF-Tu1 pass through (note that no overnight dialysis occurs). Subsequently, the solution containing EF-Tu1 was transferred to a column of Red A or Blue B Matrix gel (Amicon), which was eluted with a 0–1 M NaCl gradient, EF-Tu1 appearing around 450 mM NaCl. For the isolation of the *tuf-3* product from *E. coli* see Results.

Freeze-sweeping. Proteins of the cell extracts were submitted to 2% agarose gel electrophoresis in 100 mM Tris/borate buffer, pH 8.5. Part of the gel was stained with Coomassie Brilliant Blue to visualize proteins. The *tuf-3* gene product was cut from the unstained gel and transferred to small tubes, containing siliconized glasswool, and with a hole in the bottom. The tubes were kept at -90 °C for 60 min, whereafter the protein was isolated by centrifugation (2000 g, 15 min, at room temperature).

Preparation of antibodies. New Zealand White rabbits were immunized with 10–100 µg purified *tuf-1* or *tuf-3* gene product and bled as described previously (Heinstra *et al.*, 1986). Sr.EF-Tu1 from *S. ramocissimus* and Sr.EF-Tu1 and Sr.EF-Tu3 purified from recombinant *E. coli* were used.

SDS-PAGE and immunoblotting. After SDS-PAGE on a 10% polyacrylamide gel according to Laemmli (1970) proteins were blotted to 0.45 µm pore-size nitrocellulose sheets by an electrophoretic transfer for 1 h at 150 mA and 4 °C. The blots were washed twice in TBST (150 mM NaCl, 100 mM Tris, 0.5% Tween-20, pH 8.0) and treated with 1% ovalbumin in TBST for 1 h at room temperature. The blots were then incubated overnight at 4 °C in TBST with 0.5% (v/v) human serum and with a 1:2000 diluted antibody preparation. Human serum was replaced by 5% (w/v) non-fat milk if non-specific background signals had to be reduced. After rinsing in TBST, the blots were incubated for 30 min at room temperature with 2000-fold diluted alkaline-phosphatase-conjugated goat antirabbit IgG in TBST. Blots were finally developed by staining with bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

RESULTS

Isolation of three *tuf*-like genes from *S. ramocissimus*

A Southern blot of *S. ramocissimus* chromosomal DNA digested with either *Bam*HI, *Bgl*II or *Pst*I revealed three bands ranging from 2.8 to 15 kb when hybridized with a *E. coli tufA* probe (Fig. 1). Two bands can be explained by a restriction site within a *tuf* gene with an expected size of about 1200 bp but three bands cannot. Apparently, at least two separate *tuf*-like genes are present on the *S. ramocissimus* genome. Fragments were therefore cloned and sequenced for further identification.

Using a fragment completely internal to the *E. coli tufA* coding sequence (*Hpa*I–*Nru*I) as a probe and *E. coli* as the host for cloning, a high background from the chromosomal genes was to be expected when screening libraries by colony hybridization. Therefore we applied a sib-selection procedure in which Southern hybridization was used to screen plasmid DNA isolated from transformant pools. By successively reducing the pool size the 2.8 kb *Bgl*II fragment, which hybridizes strongly with the *tufA* probe (cf. Fig. 1), was cloned. The DNA sequence was determined and analysed by computer with the Wisconsin

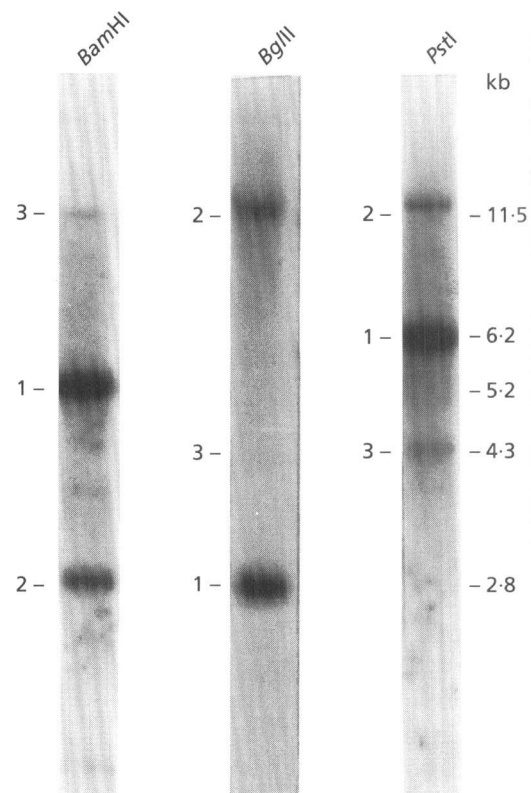


Fig. 1. Southern blot of *S. ramocissimus* chromosomal DNA. DNA was digested with *Bam*HI, *Bgl*II or *Pst*I and probed with the *Hpa*I–*Nru*I fragment of *E. coli tufA*. The *tuf* gene identified on each of the signals is indicated with the corresponding number. The signal for *tuf-3* is not visible in the *Bgl*II lane but its position was identified on a Southern blot probed with the *tuf-1* gene of *S. ramocissimus* (data not shown).

(a) *tuf-1*

1 ATCTACCGCATGCACGCCAACAGCGTGAGGAGATCGAGTCCGGTGGCGCCGGTGCACATCGTCCCGTCAATGGGCCGGAAGCAGACCACCACCGGTGAGA
 I Y R M H A N K R E E I E S V G A G D I V A V M G L K Q T T T G E T

101 CGCTGTCCGACGACAAGACCCGGTGATCCGAGCTCCATGGACTTCGCGGCCCGGTCATCCAGGTCGCCATCGAGCCCAAGTGAAGGGCGACCAGGA
 L S D E K S P V I L E S M D F P A P V I Q V A I E P K S K G D Q E

201 GAAGCTCGCGCTCGGATCCAGCCGCTGGCCGAGGAGGACCCCTCCCTCCAGGTCACATCGACGAGGAGACCGCCAGACCATCATCGCGGCATGGGC
 K L G V A I Q R L A E E D P S F Q V H S D E E T G Q T I I G G M G

301 GAGCTGCACCTCGAGGTGCTGGTCCGACCGTATCGCCGCTGAGTTCAGGTCGAGGCCAACGTCGGCAAGCCGCGAGGTCGGCTACCGCGAGAGCATCCGTC
 E L H L E V L V D R M R R E F K V E A N V G K P Q V A Y R E T I R Q

401 AGGCCGTCGAGAAGGTCGAGTACACCCACAAGAAGCAGACGGGTGGCCCGGCCAGTTCGCTCGCGTCATCATCGGCATCGAGCCATCGAGAGCGGGCA
 A V E K V E K V T G G R V P K E Y I P S V D A G A Q E A M Q F G

501 CACGTCGTACGAGTTCGTCAACAAGTCCACCGTGGCCGCTGCGAAGGAGTACATCCCTCGGTCGACGCGGTGCGCAGGAGCCATGCAGTTCGGC
 T S Y E F V N K V T G G R V P K E Y I P S V D A G A Q E A M Q F G

601 ATCCTCGCGGTTCAGAGATGACCGGTGTCGCGTACGCTGCTCGACGCGCCCTACCACGAGGTCGACTCCCTCCGAGCTCGCGTTCAGATCGCGGTT
 I L A G Y E M T G V R V T L L D G A Y H E V D S S E L A F K I A G S

701 CGCAGCCCTTCAAGGAGCCGCGCGGAAGGCCAAGCCCTGCTCTCTGAGCCGATGATGGCCGTCGAGGTCACCACGCCGAGGACTACATGGGTGAGT
 Q A P K E A A R K A K P V L L P P M M A V E V T T P E D Y M G E V

801 CATCGCGCATCAACTCCCGCGTGGCCAGATCCAGGCCATGGAGGAGCGGCGCGGTCGCGCGCTCGTGAAGGGTCTCGTCCCTCCGAGATGTT
 I G D I N S R R R A G Q I Q A M E E R A G G A R V V K G L S E M F

901 GGTTACGTCGGGACCTCGCAGCAAGAGCTCCGCGCGGACGCTACCTGCACTTCGACTCCTACCGCGAGGTTCCCGGAACGTCGCCGAGGAGA
 G Y V G D L R S K T S G R A S Y S M Q F D S Y A E V P R N V A E E I

1001 TCATCGCGAAGGCCAAGGGCGAGTAAACCCACCGCTTCACCGCGTAGGCTTGACTCCGGAGTTCGCGGGCCATTCATCCATATGCGGGTGAATGG
 I A K A K G E *

1101 CCGCGGGCCCGGACTTAAACAGCAAAGATCACCTGGCGCGATTCGAACCAAGGCGTACAGAACCACTCCACAGGAGGCCCCAGTGGCGAAGGGCAAGT
 A K A K F

1201 CGAGCGGACTAAGCCGACGTCACATCGGCACCATCGGTACATCGAATCAGGTAAGACGACCCCTCACGGCCGCCATTACCAAGGTGCTGCACGACCGG
 E R T K P H V N I G T I G H I D H G K T T L T A A I T K V L H D A

1301 TACCCGACCTGAAGGAGGECACCCCGTTCGACAACATCGACAAGGCTCCTCGAGGAGCGGTCAGCGCGGTATCACCATCTCCATCCGCGCAGCTCGAGTACC
 Y P D L N E A T P F D N I D K A T P E R Q R G I T I S I A I E P I E S G D

1401 AGACCGAGGCGGCTCACTACGCCACGTCGACTGCGCGGCTACCGGACTACATCAAGAACATGATCAGGGTGGCGCGAGATGGACGGCGCCATCTCT
 T F A R H Y A H V D C P G H A D Y I K N M I T G A A Q M D G A I L

1501 CGTGGTCGCGCCACCGACCGCCCGATCGCGCAGACCAAGGAGCAGCTGCTCCTGGCCCGCCAGGTCGGCGTTCGCTACATCGTGGTCCGCTGAACAAG
 V V A A T D G P M P Q T K E H V L L A R Q V G V P Y I V V A L N K

1601 GCCGACATGGTGGACGACGAGGAGATCATGGAGCTCGTGGAGCTCGAGGTCGCTGAGCTCCTCTCCGAGTACGAGTTCGCGGGCAGCAGCTGCGGGTGG
 A D M V D D E E I M E L V E L E V R E L L S E Y E F P G D D L P V V

1701 TCCGCTCTCCGCGCTGAAGGCGCTGGAGGGCGAGCTCAGTGGAGCGAGTCCGCTCCGACCTGATGAAGGCGGTCGACGAGTCCATCCCGGAGCCGGA
 R V S A L K L E G D A Q L E G V S V L D L M K A V D E S I P E P E

1801 GCGCGACCTGACAAGCCGTTCTCTCATGCGGATCGAGGACGCTTTCAGTACACCGGTCGCGGCACGGTCCGTCACCGGCGGATCGAGCGTGGTTCCTG
 R D V D K P F L M P I E D V F T I T G R G T V V T G R I E R G V L

1901 AAGGTC AACGAGACCGTCGACATCATCGGATCAAGACCGAGAAGACCAACCCGTCACCGGCATCGAGATGTTCCGCAAGCTGCTCGACGAGGGCC
 K V N E T V D I I G I K T E K T T T T V T G I E M F R K L L D E G Q

2001 AGGCCGTTGAGAAGCTCGGCTGCTGCTCCCGGCATCAAGCGGAGGACGTCGAGCGCGCCAGGTCATCATCAAGCCGCGCTCGGTACCCCGCACAC
 A G E N V G L L L R G I K R E D V E R G Q V I I K P G S V T P H T

2101 CGAGTTCGAGGCGCAGGCCATACCTCTCTCAAGGACGAGGGTGGCGGCCACCGCGTTCCTTCAACAACACTACCGCCCGAGTTCCTACCTCCGTACCAGG
 E F E A Q A Y I L S K D E G G R H T P F P N N Y R P Q F Y F R T T

2201 GAGCTGACCCGCGTGTGACCTCCCGAGGGCACCAGATGGTCACTGCGCGGCGACAACCCGAGATGCGCGCTCGAGCTGATCCAGCCGCTCGCCATGG
 D V T G V H L P E G T E M V M P G D N T E M R V E L I Q P V A M E

2301 AGGAGGGCCGTAAGTTCGCCATCCGTGAGGTTGGCCGACCGTCCGCGCGCCAGGTCACCAAGATCGTCAAGTAAGTTCGCGTTCGTTGAGGGTCGGG
 E G L K F A I R E G G R T V G A G Q V T K I V K *

2401 TCTGACCAAGGCTGTGACCTCAAGGGGCGGTACGACTGCGGTGTCGCGGGCCCTTTGCATGTGCGGTGTCATCAGGTGAACCTCTGGCGAACCCCTG
 2501 CCGCGGGGCGCGTGTGACGCTGTGGCGGCATAGAGTGAAGCAGATGTTCCGCGCAGGTCGCCGCTCGCAGCTCCGCGCCCGCCCGCTGCCCCCGCT
 2601 CGTCCGCTCCCGCGCAAGCCCGCGCCGAGGCCCGCTTCGCGCGCGCAACGCTCCCGCTGTACGCGGTCGACGGCATCCGGCTGCTCGCGCCCTG
 2701 ATCGTCGCGCTGACCACTACCGCGGCAACCGCGGCGGCAACCGGATCTGGGGCGCGCGGCTCCGAGATCATGCGCAGCAGCTAC
 2801 ACGTGGCCAGTACCGCTGGATCGCGCTCGAGATCT

Fig. 2. DNA sequences of (a) *tuf-1*, (b) *tuf-2* and (c) *tuf-3* from *S. ramocissimus*. The deduced amino acid sequences are indicated below the DNA sequence and the putative ribosome binding sites (AGGAGG for both *tuf-1* and *tuf-2*, and GAGG for *tuf-3*) are underlined. Nucleotides 1-1026 in (a) code for the C-terminal part of EF-G. The nucleotide sequences are deposited in the EMBL Data Library, accession nos X67057 (*tuf-1*), X67058 (*tuf-2*) and X67059 (*tuf-3*).

GCG programs Translate and Codonpreference (Deveraux *et al.*, 1984) which revealed two open reading frames (ORFs) (Fig. 2a). One ORF displayed a high degree of

identity with the C-terminal part of the *E. coli fus* gene, encoding EF-G (Zengel *et al.*, 1984) and the other with *tufA* (Yokota *et al.*, 1980). The latter ORF codes for a

(b) *tuf-2*

1 GGATCCCGGCGAATCATCCACGAGCACCAGGACGATCGCGGGACCACTTCGCCAGCCGGGACGCGCCGAGGCCGGCGGTCGGGGCGTCTT
 101 GACGGGAAGTCGTCATGTCGACGGTCTCTCTCTGTTTCACACCGATTCGCCGCCAACCGCGGGCCAGACAGCGCTGTTTCGAACTCCATGCGCACAAACG
 201 CGTGTCCGCGGGCGCTGTTTCGCGACGGGTGGAGCGAAATACACACCAAGCTGGAGCCAAATACACAGGAAGAGGACACGGCATCCCGGGATCACCCG
 301 AGGTGACAGGAGGCGCGCGAGTGGCGAAGGCGAAGTTCAGCGGACCAAAACCGCACCTCAACATCGGACCCATCGGACCCAGGCAAGACCGA
 A K A K F Q R T X P H V N I G T I G H I D H G K T T
 401 CACTCAAGTCGCGCATACGAAGTGGTGCAGACCGGTTCCCGGACCTCAACCGGTTACCCCGTTCGACCGAGATCGACAAGGCGCCGAGGAACCGCA
 L T A A I T K V L A E R G S G T P V P P D R I D H A P E E A A R G
 501 GCGCGGATCACCATCTGATGTCGCTACGTTGAGTACACAGACCGAGCCGGCACTACCGCACGTCGACTGCCCCGACACCGGACTACATCAAGAAG
 R G I T I S I A H V E Y Q T E A R H Y A H V D C P G H A D Y I K N
 601 ATGATCACCGGCGCGCCAGATGACGCGCGGATCTGGTCTGTCGCGGCAACCGGCGCGGATGCCCCAGACCAAGGAACATGTGCTGCTGGCACGGC
 M I T G A A Q M D G A I L V V A A T D G P M P Q T K E H V L L A R Q
 701 AGGTGGCGGTGCGCTACATGCTGCTGCGCGTGAACAAGACCGACATGGTCGACGACGAGGAGATCTCGAACCTGCTGGAGTTGGAGTTCGGGAGCTGCT
 V G V P Y I V V A L N K T D M V D D E E I L E L V E L E V R D L L S E H
 801 CACCGACTACGAGTTCGCGGGGACGACGTCGCGGTGCTCAAGGTGTCGGCGCTCAAGGCCCTGGAGGGCGACCCCGGTGGACCGCGTCTGGTACTCGAA
 T E Y E F P G D D V P V V K V S A L K A L E G D P R W T R S V L E
 901 CTCTCGACCGCGTCGACGACTTCGTCGCGGAGCCGGTTCGGGACGTCGACCGCGCTTCCTGATGCGGATCGAGGACGCTTTCACATCCCGGACGGC
 L L D A V D P V P E P V D R P P F L M P I E D V F T T I T C R G
 100: GCACCGTCTGTCACCGCGCGGATAGAGCGGGCACGCTGAACGTGAACACCGGAGTGGAGATCATCGGCATCCACGAACAGAGACCGGACCCGGTCTAC
 T V V T G R I E R G T L N V N T E V E I I G I H E Q R T R T T V T
 110: CGGCATCGAGATGTTTCGCAAGCTCTCGACGAGGTCGCGGCGCGGAGAACGTCGGACTGCTGCTGCGCGGAGTGAAGCCGGACAGGTCGAGCGCGGT
 G I E M P R K L L D E G R A G E N V G L L L R G V K H E Q V E R G
 120: CAGGTGCTCATAGCCCGGGTCCGTCACCCGCAACCGAGTTCGAGCGCGAGCGGTACATCCCTGTCGAAGGACGAGGGCGGGCGGCACACCGCTTCT
 Q V Y I P H T Q P E A Q A Y I L S K D E G G R H T P P F
 130: TCGAGAATACCCCTCCGCGATTCTACTTCGCGACCCAGCTCACCGGCGTGGTGAAGCTGCGGAAGGGCAACGAGATGTTGATGTCGCGGCGCAACAC
 E N Y R P Q F Y P R T T D V T G V V T I P K G T E M V M P G D N T
 140: CGCCATGCAAGTCCAGCTGATCCAGCCATCGCCAATGGAGGAGGGGCTGAAGTTCGCGCATCCGCGGAGGGCGGGCGCACGTCGCGCGCGCGCGAGTCAAG
 A M H A I V Q P I A M E E G L K F A I R E G G R T V V G A G G V T
 150: CGGATCGTAAGTAGCCCTGCTCGACCGGAAACAGCCGCTGCGGGGTGAACAGCCCGCTGATAAGGAGCTGACGCGGGCTCGGGCTGGCCAGG
 R I V K *
 160: GCGCGCTCAGGCTCGGCGCGGGCGGACGAGCCGGTCAAGCCGTCGGCGCGAGACCGGACTAACGATGGTGCACACGA

(c) *tuf-3*

1 GCGGCGCGCGCCAGGACCGTTCGCGTGGAGGATCGCGACCGGACGCTCGAGCCGAACAGCGGCGCCGCGCGCGAGTTCCAGCAGCGCGCGCGGACGA
 101 AGCCACAGGTCGACAGCGGTACGCGCACCGCAACACCGCGCGGCTGTCAGCCCGCCAGGTCGGGCGGCGAGCCGTCGGTGCAGCAGCGCGTGGCGGT
 201 CGAAACAGGACACACAGGACCGCGCGCGCGGAGCAGCGCGGACAGGAGAAATCGGACAGGCGACAGCGCGGTCGACGCGCGCGCGGCGGAG
 301 CCGGACCGGCGACCGCGCGCGCGCGGAGCGCGCGCGCGGTCATGACCGGTGAGCTCGCTAAAGCGCGAGCTACGTCGCGCGCGCGCGCGCGGCG
 401 CCGCAGAGCGGTCGCGCGCGCGCGGATCGAGCATCCGCGGACACTAAACCGGATGACATGTCGATCGCGGTCATGGTTTCGCTCATGACCGCGCGGTA
 501 CGGTGGGTCACCGCGCGCGCGCGTCCGCGACAGGACCCCGCTGCGCGCGCGCGCTTCGCGCGCGCGCTTCGCGCGCGCGCGCGCGCGCGCGCGCG
 601 CTCACGGCTTCGCGCGCGCGCGCGGTCGCGCGCGCGCGGAGTACCGAGTCCGCGCGCGCGCTTCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
 701 TCTGGCGCGCACATGTCGCGCGCGCGCGCTGTCG
 801 TACCGCGCTTCG
 901 GCAAAAGCGTCG
 100: GCGCGCATTCGACCG
 110: TAGAGACTTCAGGTCACGCGCATGTCGAAGCGCGCAATGTCG
 S K T A Y V R T K P H L N I G T M G H V D H G K T T
 120: CGTTGACCG
 L T A A I T K V L A E R G S G T P V P P D R I D H A P E E A A R G
 130: CATCACCATCAACATCG
 I T I N I A H V E Y E T D T R H Y A H V D M P G H A D Y V R N M V
 140: ACCG
 T G A A P Q L D G A I L V V A A T D G P M P Q T A E H V L L A R Q
 150: GCGTCGACACATCGCTGTCG
 V D H I V V A L N K A D A G D E E L T D L V E L E V R D L L S E H
 160: GCGCTACCG
 G Y G R V S G L K A L E G D P K W T A S I E A L L G
 170: GCGGTGGACACCTACGTCGCGATGCGCGGAGCGGATGTCGACG
 A V D T Y V P M P F R Y V D A P P L L P V E N V L T I T G R G T V V
 180: TCACCGGAGCGTCGAGCGGGCG
 T G A V E R G T V R V G N R V E V L G A G L E T V V T G L E T F G
 190: CAAGCCATGACGAGAGCG
 K P M D A G A G D N V A L L R G V P R D A V R R G H V V A A P
 200: GGGAGCGTGGTTCG
 G S V V P R S R F S A Q V Y V I S A R E G G R T P V T S G Y R P Q
 210: AGTTCTACATCCCTACCGCGATGTCGTCGGGAGCGTCGACCTGGGAGGTCGGGGTCTGCTCGCGCTGGGGAGACGGTTTCGATGTCGCGACTCGG
 F Y I A R D I R V G D V L G G V G V A R P G E T V S M I V E L G
 220: CCGGAGGCTTCG
 R E V P L E P G L G F A I R E G G R T V G A G T V T A L V *
 230: ACCG
 CATCCCTCTCCGTCGCGTGAATTCGCGGACTCCCAAGCTGACG
 240: TCTACGTCGATTCGACGACCG
 250: CTGCTACACTCGGTCGGGTCGCTGCACTTCG
 260: CGTGTACACTCGGTCGGGTCGCTGCACTTCG
 270: CTGGAGCTGGTCGCGAAGTTCGCGCTTC

protein of 396 amino acids, designated (Sr.)EF-Tu1 (see Fig. 3). A probe derived from the C-terminal part of the *fus* gene present on the cloned *Bgl*II fragment gave a single signal in the Southern blot. This probing also showed that the 2.8 kb *Bgl*II fragment is part of the 5.2 kb *Bam*HI and of the 6.2 kb *Pst*I fragment (data not shown). Partial sequencing of the 6.2 kb *Pst*I fragment and hybridization studies suggest that the gene organization on this part of the *S. ramocissimus* genome is *rpsL-rpsG-fus-tuf*, identical to that of *E. coli* (Post *et al.*, 1980), *Spirulina platensis* (Buttarelli *et al.*, 1989), *Micrococcus luteus* (Ohama *et al.*, 1987) and *Anacystis nidulans* (Meng *et al.*, 1989) (data not shown). To gain more insight into the nature of the fragments that hybridized more weakly with the *E. coli tufA* probe (*Bam*HI fragments of 2.8 and 12 kb and *Pst*I fragments of 4.3 and 15 kb), the Southern hybridization experiment was repeated with probes from the N-terminal and C-terminal part of the *S. ramocissimus tuf-1* gene. Both probes hybridized with each of the four fragments, indicating that they contain complete *tuf*-like genes.

A *tuf-1* probe that gave a stronger signal in the Southern analysis than the *E. coli tufA* probe was used to clone the 2.8 kb *Bam*HI fragment and a 9 kb *Eco*RI-*Bam*HI fragment. The 2.8 kb *Bam*HI fragment carries an ORF corresponding to a protein of 396 amino acids, whereas the 9 kb *Bam*HI-*Eco*RI fragment carries an ORF corresponding to a protein of 388 amino acids (Figs 2b, c, 3). The genes were designated *tuf-2* and *tuf-3* and their products Sr.EF-Tu2 and Sr.EF-Tu3, respectively, although we do not know whether they encode genuine EF-Tus.

Nucleotide and amino acid sequence comparisons

Degrees of nucleotide sequence identity of the three *S. ramocissimus tuf*-like genes and *E. coli tufA* are listed in Table 1. Most strikingly, the *S. ramocissimus tuf* genes display a much lower degree of similarity than do the two *E. coli tuf* genes (99%), which differ at four base positions only. The nucleotide sequence of *tuf-3*, encoding the GDP-binding domain (amino acids 1–200), has 76% identity with the corresponding sequences of *tuf-1* and *tuf-2*. The identity drops to 63%, however, for the sequence encoding the C-terminal part of the protein. This drop in identity coincides with a change in the codon usage towards favouring codons with A or T in the third

position, which is unusual for *Streptomyces*. Obvious deviations from the codon usage often found in genes with a high level of expression (Wright & Bibb, 1992) are not observed in *tuf-1* and *tuf-2*. However, it is noteworthy that *tuf-1*, but not *tuf-2* and *tuf-3*, has a high percentage of GGT codons for Gly, as has also been observed for the ribosomal protein L7/L12 of *S. antibioticus* (Parra *et al.*, 1992).

Both *tuf-1* and *tuf-2* are preceded by putative ribosome binding sites with 6/6 homology to the 3' end of known 16S rRNA sequences (van Wezel *et al.*, 1991, and references therein). In the case of *tuf-3* this fit is only 4/6 (Fig. 2c). A search for promoter sequences upstream of the three *tuf* genes using the *Streptomyces* promoter consensus sequence described by Strohl (1992) was unsuccessful. Sequences upstream and downstream of the genes were analysed for identity with known sequences in the databases. A positive result was obtained only in the case of the *tuf-1* upstream region where the *fus* gene, encoding EF-G, was localized.

In Fig. 3 the deduced amino acid sequences are compared with the *E. coli* EF-Tu sequence since most of the data regarding structure/function relationships concern the *E. coli* protein. All three *Streptomyces* proteins contain the consensus sequence typical for GDP-binding proteins (Dever *et al.*, 1987) and show a 100% fit with the P-loop motif (Saraste *et al.*, 1990). The Ec.EF-Tu amino acid residues His-66, Lys-208 and Lys-237 (underlined in Fig. 3) that have been cross-linked to tRNA (Duffy *et al.*, 1981; van Noort *et al.*, 1984) are conserved in EF-Tu1 but not in EF-Tu2 or EF-Tu3. Lys-56 of Ec.EF-Tu, which has been found to be methylated in the late exponential phase and stationary phase (van Noort *et al.*, 1986), is not present at that position in any of the three. All amino acid residues known to be altered in kirromycin-resistant mutants are conserved in the three Sr.EF-Tus (see Discussion). On the other hand, Lys-357 of Ec.EF-Tu, previously cross-linked to kirromycin (van Noort *et al.*, 1984) is not conserved.

The results of a comparison between the deduced amino acid sequences of the three Sr.EF-Tus and some of the known EF-Tu sequences of almost all the phyla in the eubacterial kingdom, one representative of the archaeobacteria and two eukaryotes are shown in Table 2. Sr.EF-Tu1 and Sr.EF-Tu2 are more closely related to each other than to any of the other sequences listed in Table 2. This suggests that the genes were derived relatively recently from a common ancestral gene. In contrast, the similarity between EF-Tu3 and EF-Tu1 or EF-Tu2 is only slightly higher than with any of the other eubacterial EF-Tus. The degree of identity of EF-Tu3 to each of these other eubacterial EF-Tus is about the same, but much higher than to the archaeobacterial or eukaryotic EF-1 α species. So, although a relationship of EF-Tu3 with the EF-Tus from the same organism is not apparent, EF-Tu3 clearly belongs to the eubacterial lineage. This is consistent with the finding that the three Sr.EF-Tus show the same degree of identity/similarity with the EF-1 α species of *Rhizomucor racemosus* and *Xenopus laevis*. Interestingly, EF-Tu3 shows a somewhat closer relationship with *Thermo-*

Table 1. Nucleotide sequence comparison of the three *S. ramocissimus tuf*-like genes and *E. coli tufA*.

	% identical		
	<i>tuf-1</i>	<i>tuf-2</i>	<i>tuf-3</i>
<i>tuf-1</i>		85	70
<i>tuf-2</i>			70
<i>tuf A</i>	71	69	63

```

EF-Tu1  1 AKAKFERTKPHVNIGTIGHIDEGKTTLTAAITKVLHDAYPDLNEATPFDN
EF-Tu2          Q                               RF   PF   Q
EF-Tu3  S TAYV          L   M   V           .. ERGSGTFV   R
EF-TuA   S E           V   V           T AKT GG..A RA Q

51 IDKAPEERQRGITISIAHVEYQTEARHYAHVDCPGHADYIKNMITGAAQM

      R   AA   N   E DT   M   V   V   L
      N   KA  NTS  D PT           V

101 DGAILVVAATDGMPQTKEHVLLARQVGVPYIVVALNKADMVDDEEIMEL
          S L I   A           DH           .AG   LTD
          R I G           I F C           LL

151 VELEVRELLSEYEFPGDDLPVVRVSALKALEGDAQWTQSVLDLMKAVDES
          T           V K R   PR R E LD F
          D   HGYG GA   G   PK A IEA LD TY
M           Q D   T I G           E EAKI E AGFL SY

201 IPEPERVDQKPFLMPIEDVFTITGRGTVVTGRIERGVLKVNETVDIIGIK
V V   R           T N TE E H
V M   Y A   L V N L           AV   TVR GNR EVL AG
          AI   L   S S           V   II G E E V

251 TEKTTTTVTGIEMFRKLLDEGQAGENVGLLLRGIKREDVERGQVIIKPGS
EQR R           R           V Q   V R
L ... V L T G PM A D A   VP DA R H VAA
. TQKS C V           R   V           EI   LA T

301 VTPHTEFEAQAYILSKDEGGRHTPPFNNYRPQFYFRTTDVTGVVHLPEGT
          Q           E           T K
V RSR S V V AR   T VTSG   I A V D D G .V
IK K SEV           KG           TIE V

351 EMVMPGDNTEMRVELIQPVAMEEGLKFAIREGGRTVGAGQVTKIVK 396
          A H Q   I           R 396
GVAR ETVS I GRE PL P G           T AL . 388
          IK V T H I DD R           V A VLG 393

```

Fig. 3. Amino acid sequences of EF-Tu1, 2 and 3 from *S. ramocissimus* and EF-TuA from *E. coli* aligned with the GAP program of the Wisconsin GCG sequence analysis package (Devereux *et al.*, 1984). The GDP binding consensus sequence is underlined and in bold. The residues corresponding to the Ec.EF-Tu amino acids involved in tRNA interaction are underlined in the EF-Tu1 sequence. The amino acids changed in kirromycin-resistant *E. coli* EF-Tus are indicated in bold. The residues Lys-56 and Lys-357 which are sometimes methylated in EF-TuA are printed in italic and underlined.

coccus celer EF-Tu than do EF-Tu1 and EF-Tu2. The unusual sequence data of EF-Tu3 prompted a search for *tuf-3* homologues in *Streptomyces* spp. and other bacteria.

Number of *tuf*-like genes in other *Streptomyces* species

The presence of three *tuf*-like genes in *S. ramocissimus* raised the question whether this is a particular feature of this species or a more general gene arrangement of *Streptomyces*. We therefore analysed chromosomal DNA of several *Streptomyces* species digested with *Pst*I and *Bam*HI by probing the Southern blot with a fragment internal to the coding sequence of *tuf-1* (Fig. 4a), of *tuf-2*

(Fig. 4b) and of *tuf-3* (Fig. 4c). The signals in (a) correspond to *tuf-1* homologues in the various strains. The signals in (c) are *tuf-1* [cf. (a)] and *tuf-3* homologues. In addition some weaker signals can be observed which have not been characterized further. The *tuf-2* probe in (b) does not reveal *tuf-2* homologues in any of the strains except for *S. ramocissimus*. The stronger signals correspond to *tuf-1* [cf. (a)] and the weaker signals to *tuf-3* [cf. (c)].

Heterologous expression of the *tuf*-like genes in *E. coli*

Expression of *tuf-1*. Transformation of *E. coli* with pUSRT1-1 (Fig. 5a) did not result in good heterologous

Table 2. Amino acid sequence comparison of the three *tuf* gene products of *S. ramocissimus* with representatives of the various kingdoms.

Compare Woese (1987) for the phylogenetic tree. Amino acid sequences were retrieved from the Swiss Protein Data Library and aligned with the GAP program of the Wisconsin GCG sequence analysis package (Devereux *et al.*, 1984). References: 1, Ohama *et al.* (1987); 2, Yokota *et al.* (1980); 3, An & Friesen, 1980; 4, Seidler *et al.* (1987); 5, Satoh *et al.* (1991); 6, Bachleitner *et al.* (1989); 7, Mickel & Spremuli (1986); 8, Buttarelli *et al.* (1989); 9, Loechel *et al.* (1989); 10, Kraus *et al.* (1990); 11, Auer *et al.* (1990); 12, Sundstrom *et al.* (1987); 13, Djé *et al.* (1990); 14, Deschamps *et al.* (1991).

	% identical; % similar		
	EF-Tu1	EF-Tu2	EF-Tu3
Eubacteria			
<i>S. ramocissimus</i> EF-Tu1	100	88; 94	65; 78
<i>S. ramocissimus</i> EF-Tu2		100	64; 78
<i>Micrococcus luteus</i> (Gram-pos., high GC) (1)	79; 87	77; 87	61; 76
<i>E. coli</i> (purple bacteria) (2,3)	74; 85	72; 84	60; 77
<i>Thermus aquaticus</i> (thermophiles) (4,5)	71; 84	71; 84	63; 77
<i>Thermatoga maritima</i> (6)	70; 84	69; 84	63; 80
<i>Anacystis nidulans</i> (cyanobacteria) (7)	70; 83	67; 82	60; 75
<i>Spirulina platensis</i> (cyanobacteria) (8)	70; 82	67; 80	59; 75
<i>Mycoplasma genitalium</i> (Gram-pos., low GC) (9)	69; 82	67; 80	60; 74
<i>Cyanophora paradoxa</i> (cyanelle) (10)	68; 81	65; 80	59; 75
Archaeobacteria			
<i>Thermococcus celer</i> (11)	39; 61	38; 60	40; 63
Eukaryotes			
<i>Rhizomucor racemosus</i> (12)	33; 57	33; 57	35; 57
<i>Xenopus laevis</i> , 42Sp50 (13,14)	33; 52	33; 52	34; 54
<i>Xenopus laevis</i> , EF-1 α S and EF-1 α O (13,14)	33; 57	32; 57	34; 55

expression of *tuf-1* in cells during exponential growth (i.e. 1 and 2 h after induction). Highest expression was observed 16 h after induction, when the cells were in the stationary phase (Fig. 6). Further analyses were then performed with immunoblotting using antibodies raised against EF-Tu isolated from *S. ramocissimus*. The Western blot of Fig. 7 (lanes 1 and 2) shows that this EF-Tu migrates to the same electrophoretic position as the product of *tuf-1*, isolated from stationary phase *E. coli* cells after heterologous *tuf-1* expression. Therefore, we conclude that the EF-Tu species isolated from *S. ramocissimus* is the product of *tuf-1*. Both EF-Tu1 isolated from *S. ramocissimus* and the product of *tuf-1*, heterologously expressed in *E. coli* are able to participate in poly(Phe) synthesis *in vitro* and are inhibited by kirromycin (Fig. 8a, b). Kirromycin sensitivity (EF-Tu1) was dominant over kirromycin resistance (EF-Tu of *E. coli* strain PM1455) and therefore inhibition of poly(Phe) synthesis was observed (Fig. 8b).

Expression of *tuf-2*. For the expression of *tuf-2* in *E. coli* two recombinant plasmids were constructed, pUSRT2-1 and pUSRT2-3 (Fig. 5b). Expression of *tuf-2* in JM101 transformed with pUSRT2-1 could not be demonstrated (Fig. 7, lane 3). In overnight cultures of JM101 transformed with pUSRT2-3 which has part of the *tuf-2*

upstream sequence deleted, *tuf-2* expression was detectable (Fig. 7, lane 4). The heterologous expression of *tuf-2* was substantially lower than that of *tuf-1*. It could only be detected on Western blots and not in Coomassie Brilliant Blue stained gels. Moreover, about 80% of the *tuf-2* product was found in so-called inclusion bodies, which are spun down at 30000 g (data not shown). The expression was too low for purification, functional characterization and immunization. Although EF-Tu2 has the same number of amino acids (396) as EF-Tu1, it migrates considerably faster during SDS-PAGE (Fig. 7, lanes 2, 4 and 5), which made distinction between the two factors in *S. ramocissimus* extracts possible.

Expression of *tuf-3*. Expression of the *tuf-3* gene could not be demonstrated in JM101 cells transformed with pUSRT3-1 (Fig. 5c). Sequences immediately upstream of the putative ribosome binding site (GAGG, nt 1111-1114, Fig. 2c) of the *tuf-3* gene were deleted to remove potential transcriptional termination signals. This did not result in appreciable expression either. The ribosomal binding site of *tuf-1* was then cloned upstream of *tuf-3*, yielding the plasmid pUSRT3-3 (Fig. 5d). This resulted in very high expression of *tuf-3* in the *E. coli* strain KA797 (Fig. 9, lanes 1 and 2). More than 90% of the *tuf-3* gene product appeared to reside in inclusion bodies. We failed to alter

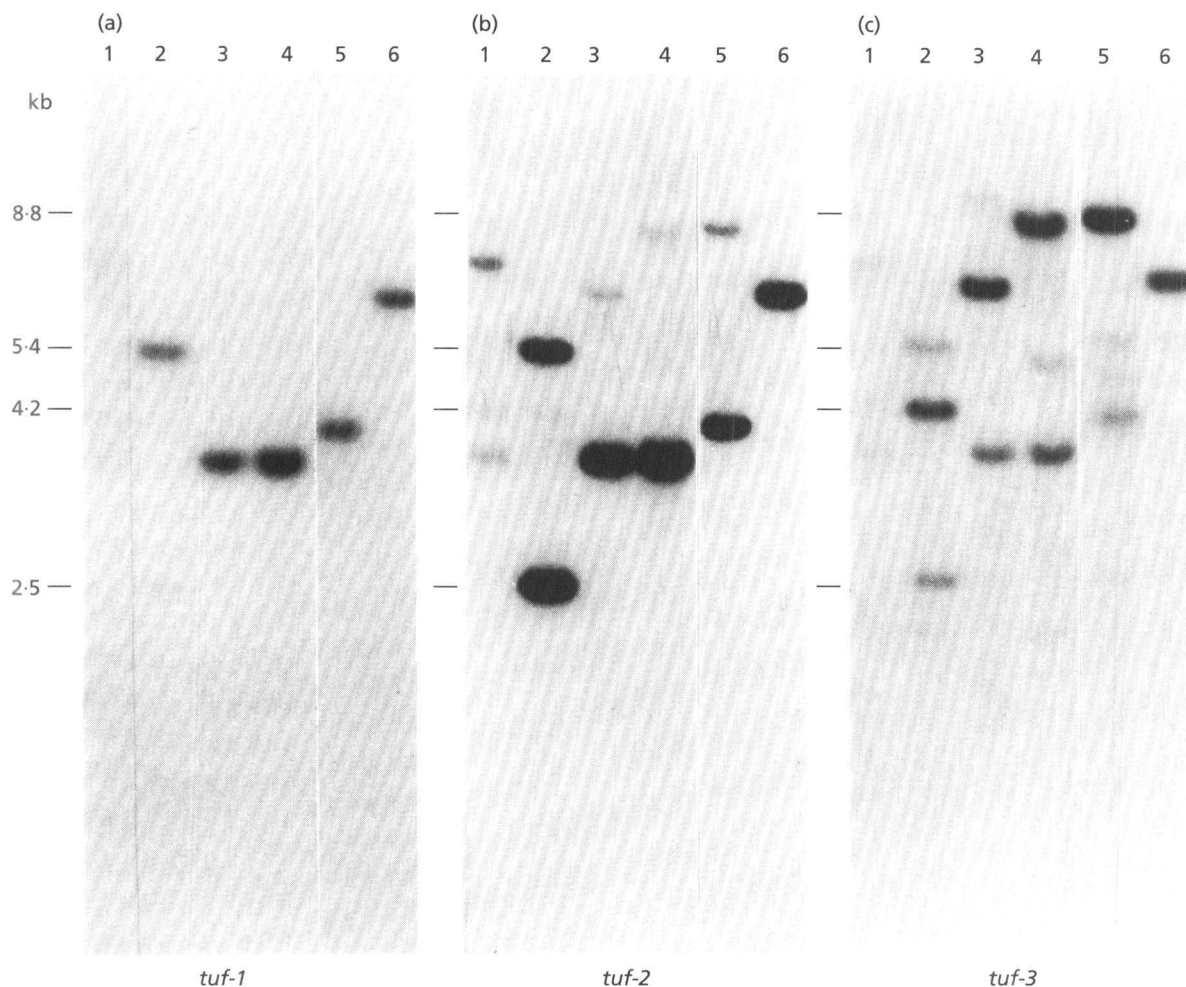


Fig. 4. Southern blot of chromosomal DNA isolated from (1) *E. coli*, (2) *S. ramocissimus*, (3) *S. collinus*, (4) *S. ambofaciens*, (5) *S. lividans*, (6) *S. glaucescens* and digested with *Bam*HI plus *Pst*I. The blots were probed with (a) 243 bp *Sma*I fragment of *tuf-1* (nt 1435–1678, Fig. 2a), (b) 1000 bp *Nar*I fragment of *tuf-2* (nt 483–1483, Fig. 2b), (c) 633 bp *Sal*I fragment of *tuf-3* (nt 1503–2136, Fig. 2c). The blot in (a) was washed under more stringent conditions ($0.3 \times$ SSC) than those in (b) and (c) ($1.5 \times$ SSC) in order to identify the signal corresponding to the *tuf-1* homologues in the various chromosomal DNAs.

this subcellular distribution of the product by varying the culturing conditions, such as lowering temperatures and shortening growth and periods of induction by IPTG. To solubilize the *tuf-3* gene product pellets were treated with 4 M urea and 100 μ g lysozyme ml^{-1} according to Frankel *et al.* (1990) and centrifuged at 30000 *g*. The *tuf-3* gene product now appeared in the S30 supernatant and remained soluble after removal of urea by passing the supernatant through Sephadex G-25 (PD10 column, Pharmacia). The eluate of this column was submitted to agarose gel electrophoresis and the *tuf-3* gene product isolated by freeze-sweeping. The *tuf-3* product thus obtained was homogeneous as judged by SDS-PAGE (Fig. 9, lane 4) and migrated slightly faster than *E. coli* EF-Tu (Fig. 9, lane 5) but significantly faster than EF-Tu1 (Fig. 9, lanes 3 and 4). Activity of the protein, however, could not be demonstrated with *in vitro* assays. Polyclonal antibodies raised against the *tuf-3* product showed cross-reactivity towards EF-Tu1 and heterologously expressed

tuf-2 product. The *tuf-3* product, however, is not recognized by anti-EF-Tu1 (data not shown).

Homologous expression

The availability of antibodies raised against EF-Tu1 enabled us to study its synthesis and that of EF-Tu2 under various conditions in *S. ramocissimus*. The antibodies raised against the *tuf-3* product enable the detection of all three EF-Tus. That they also recognize EF-Tu3 produced in *Streptomyces* was demonstrated by putting *tuf-3* behind the constitutive *ermE* promoter on the plasmid pIJ4090 (J. White & M. J. Bibb, unpublished), resulting in pISRT3-1. Over-expression had to be studied in *S. coelicolor* M145 since a suitable transformation system for *S. ramocissimus* was not found. Submerged cultures of pISRT3-1 transformants of *S. coelicolor* were grown in TSB medium for 24 h. S30 extracts were then submitted to immunoblotting, using the antibodies against the *tuf-3*

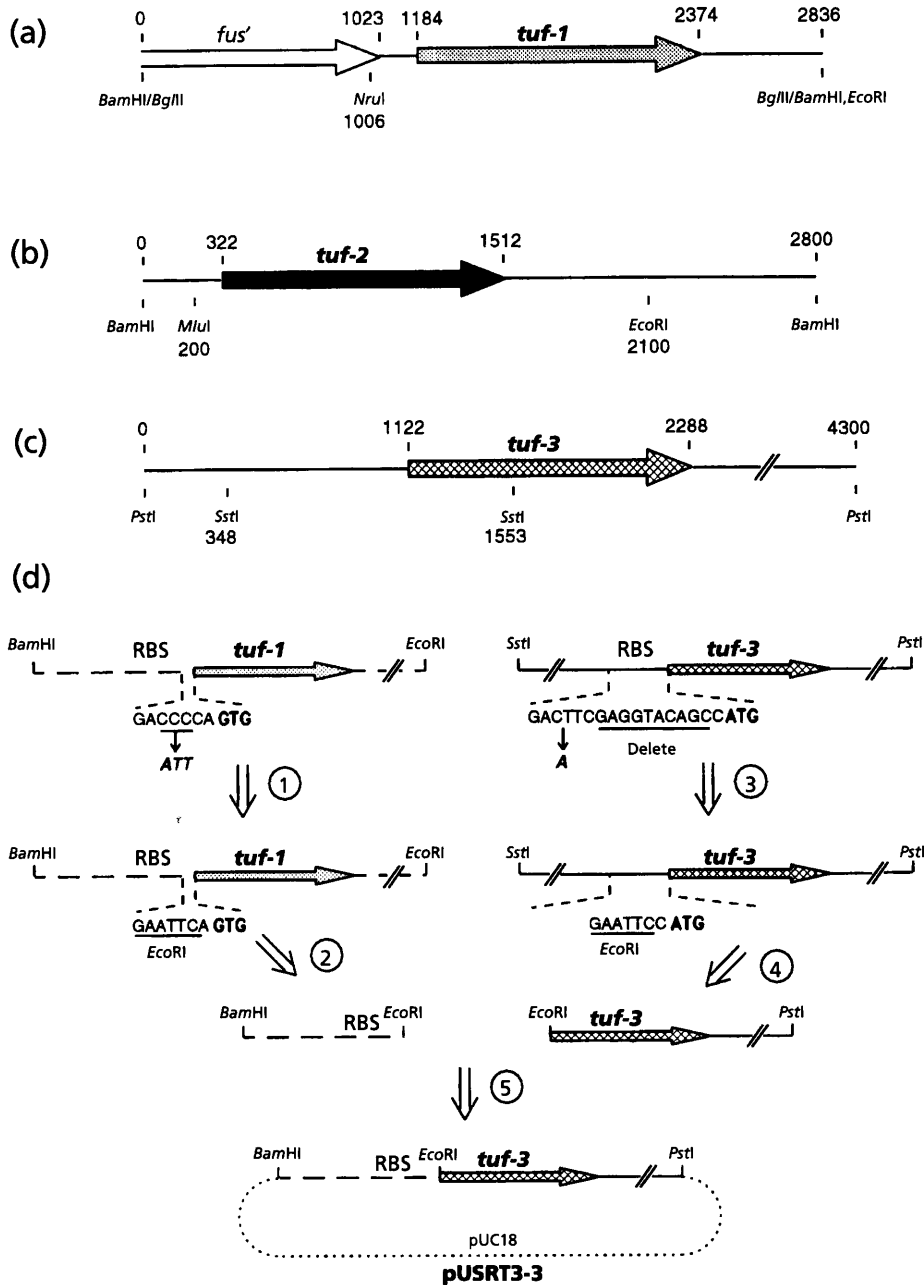


Fig. 5. Construction of plasmids for heterologous expression. (a) pUSRT1-1. A 1.8 kb *Nru*I(1006)–*Eco*RI(2836) fragment containing the entire *tuf-1* gene was subcloned from the 2.8 kb *Bgl*II fragment of pUSRT1 in *Sma*I- and *Eco*RI-digested pUC19 (Yanisch-Perron *et al.*, 1985). The *Nru*I site is located 180 bp upstream of the GTG start codon of *tuf-1* and the *Eco*RI site is from pUC19, adjacent to the *Bgl*II/*Bam*HI ligation site. (b) pUSRT2-1. From the 2.8 kb *Bam*HI fragment containing the entire *tuf-2* gene, a *Bam*HI(0)–*Eco*RI(2100) subclone was constructed in pUC19. pUSRT2-3. pUSRT2-2 was digested with *Mlu*I(200) and the protruding 3' ends were converted to blunt ends by T4 DNA polymerase. After digestion with *Eco*RI, the fragment harbouring *tuf-2* was cloned in *Sma*I–*Eco*RI-digested pUC19 resulting in pUSRT2-2. In order to ensure that translation, starting at the ATG of *lacZ* in pUC19, did not continue into *tuf-2*, the 5' protruding ends of *Hind*III (site in the polylinker of pUC19 and upstream of the *tuf-2* fragment) -digested pUSRT2-2 were filled and ligated. The plasmid pUSRT2-3 contains a stopcodon (TAG) located at the position of the original *Hind*III site and in frame with the *lacZ* start. (c) pURST3-1. This was constructed by partially digesting pASRT3 with *Sst*I and with *Pst*I. The *Sst*I(348)–*Pst*I(4300) fragment was subcloned in pUC18. (d) pURST3-3. In order to replace the ATG upstream region of *tuf-3* by that of the *tuf-1* GTG upstream region, *Eco*RI sites were constructed immediately in front of the translation start codons (steps 1 and 3). The final construct pURST3-3 was isolated after a three fragment ligation: *Bam*HI–*Eco*RI (*tuf-1* upstream region), *Eco*RI–*Pst*I (containing *tuf-3*), and *Bam*HI–*Pst*I-digested pUC18 (steps 2, 4 and 5). All base changes were made by oligonucleotide-directed mutagenesis according to Kunkel *et al.* (1990) and confirmed by sequencing.

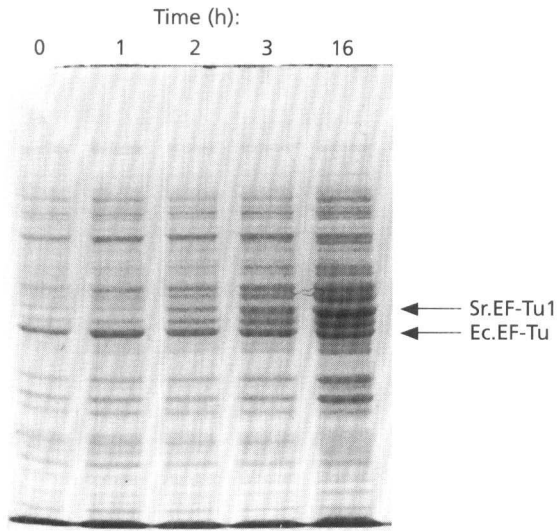


Fig. 6

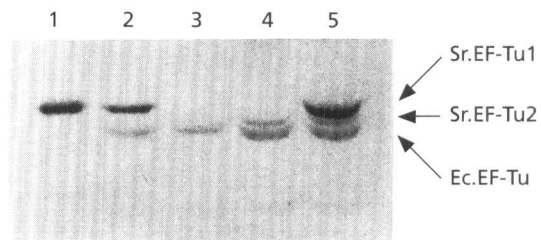


Fig. 7

Fig. 6. Temporal expression of *tuf-1* in *E. coli* JM101 studied with SDS-PAGE. *E. coli* JM101 transformed with pUSRT1-1 was grown in LB broth at 37 °C. The expression of *tuf-1* was induced by the addition of 0.5 mM IPTG at OD₆₀₀ 0.2. The indicated times are relative to the addition of IPTG (= 0 h). Total cell extracts were subjected to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The sample in lane 0 was taken immediately before the addition of IPTG. The positions of Ec.EF-Tu and Sr.EF-Tu1, as indicated in the figure, were identified in Western blots with antibodies raised against Sr.EF-Tu.

Fig. 7. Expression of *tuf-1* and *tuf-2* in *E. coli* JM101 (16 h induced with IPTG) studied with immunoblotting using antibodies raised against EF-Tu1 isolated from *S. ramocissimus*. The *tuf* gene products observed in each lane are, from top to bottom. Lane 1, EF-Tu1 purified from *S. ramocissimus*; lane 2, EF-Tu1 and Ec.EF-Tu, total extract of *E. coli* JM101 pUSRT1-1; lane 3, Ec.EF-Tu, total extract of *E. coli* JM101 pUSRT2-1; lane 4, EF-Tu2 and Ec.EF-Tu, total extract of *E. coli* JM101 pUSRT2-3; lane 5, EF-Tu1, EF-Tu2 and Ec.EF-Tu, mixture (1:1) of samples applied in lane 1 and 4.

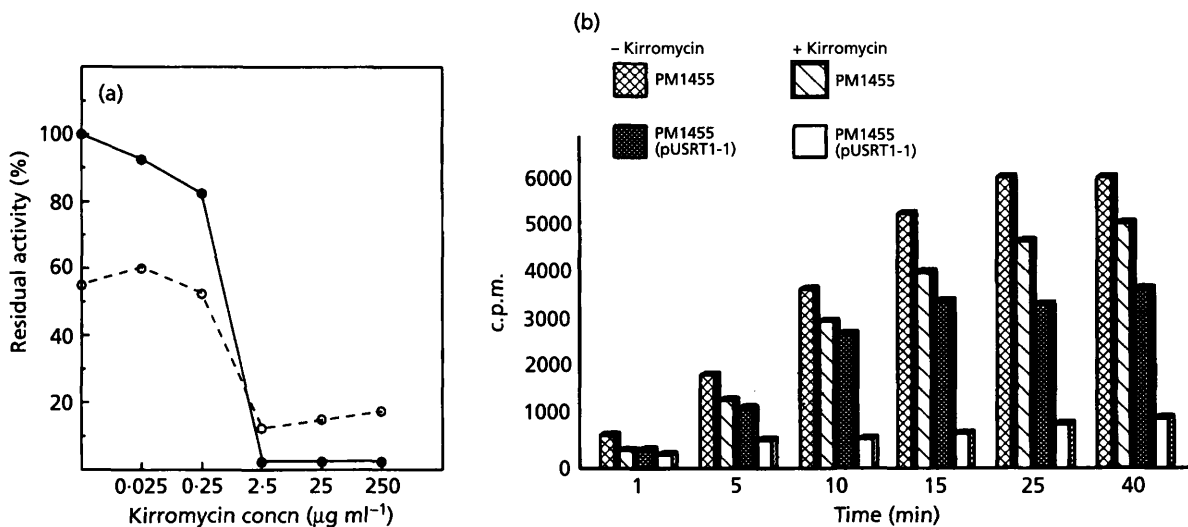


Fig. 8. EF-Tu1 is kirromycin sensitive. (a) Poly(U)-directed incorporation of [¹⁴C]phenylalanine into trichloroacetic acid precipitable material was studied as a function of the kirromycin concentration in an *in vitro* translation system dependent on EF-Tu. Reactions were carried out with ribosomes and EF-G isolated from *E. coli*. ●, Incorporation of ¹⁴C-label observed with Ec.EF-Tu (isolated from LBE1001, a wild-type *E. coli* strain) in the absence of kirromycin, normalized to 100%. ○, EF-Tu1, isolated from *S. ramocissimus*. For experimental details see van der Meide (1982). (b) Poly(U)-directed poly(Phe) synthesis was measured with and without 30 μg kirromycin ml^{-1} in S30 supernatants of the kirromycin-resistant *E. coli* strain PM1455 and in PM1455 transformed with pUSRT1-1. The S30 supernatants were fractionated over Sephadex G-25 and the first four fractions with an absorbance at 260 nm were combined. An aliquot was incubated with tRNA, [³H]Phe and poly(U) at 37 °C (for experimental details see Luiten *et al.*, 1991). Samples were taken at the indicated times and *in vitro* translation of poly(U) was measured as trichloroacetic acid precipitable ³H-labelled product.

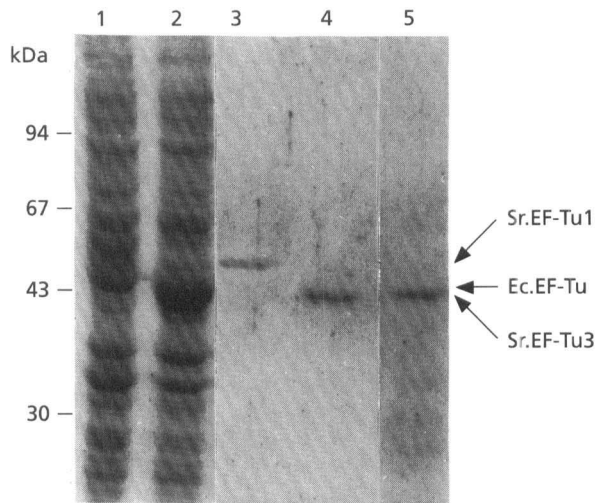


Fig. 9

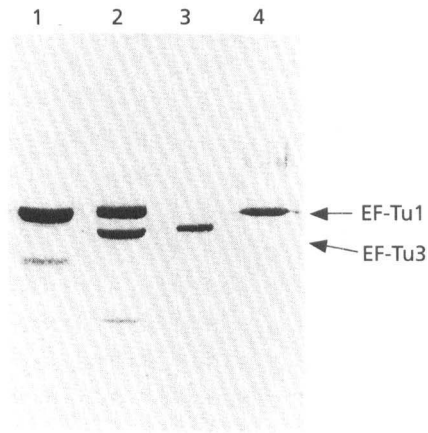


Fig. 10

Fig. 9. Expression of *tuf-3* in *E. coli* studied with SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue. Lane 1, total extract of KA797 cultured for 8 h in LB medium; lane 2, total extract of KA797(pUSRT3-3) cultured for 8 h in LB medium and induced with IPTG during the last 2 h; lane 3, EF-Tu1 purified from *S. ramocissimus*; lane 4, purified EF-Tu3; lane 5, Ec.EF-Tu.

Fig. 10. Western blot analysis of EF-Tu1 and EF-Tu3 probed with antibodies against Sr.EF-Tu3. Lane 1, Sc.EF-Tu1 – S30 supernatant fraction of liquid grown *S. coelicolor* M145; lane 2, Sc.EF-Tu1 and EF-Tu3 – S30 supernatant fraction of liquid-grown *S. coelicolor* M145 pISRT3-1; lane 3, EF-Tu3 purified from *E. coli* JM101(pUSRT3-3); lane 4, EF-Tu1 isolated from *S. ramocissimus*.

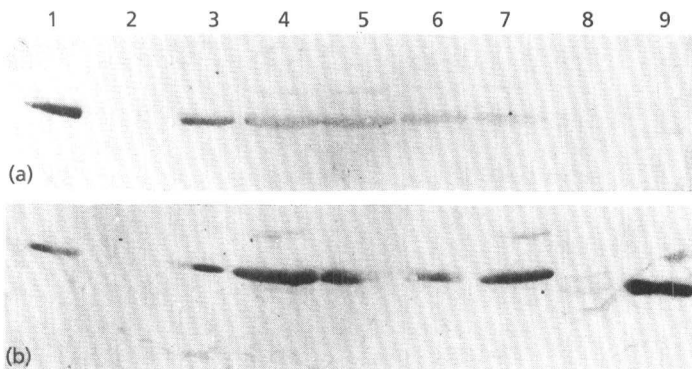


Fig. 11

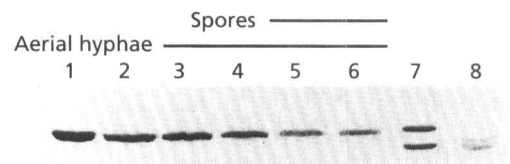


Fig. 12

Fig. 11. Temporal expression of *tuf* genes in submerged cultures of *S. ramocissimus*, studied with Western blotting using anti-EF-Tu1 (a) and anti-EF-Tu3 (b). Lane 1, purified EF-Tu1; lane 2, S30 pellet fraction after 20 h culturing; lane 3, S30 supernatant fraction after 20 h culturing; lane 4, S30 pellet fraction after 44 h; lane 5, S30 supernatant fraction after 44 h; lane 6, S30 pellet fraction after 68 h; lane 7, S30 supernatant fraction after 68 h; lane 8, EF-Tu2 and Ec.EF-Tu, total extract of *E. coli* JM101(pUSRT2-3); lane 9, EF-Tu3 purified from *E. coli* JM101(pUSRT3-3).

Fig. 12. Western blot of extracts from surface-grown *S. ramocissimus*. The blot was incubated with antibodies raised against the *tuf-3* product. In lanes 1 to 6 equal amounts of protein were loaded. *S. ramocissimus* was grown on minimal medium. The bars above the Western blot indicate the presence of aerial hyphae (42–80 h) and of spores (58–80 h) as observed by phase-contrast microscopy. S30 supernatant fractions were prepared after 18 h (lane 1), 27 h (lane 2), 42 h (lane 3), 51 h (lane 4), 66 h (lane 5), and 80 h (lane 6) of growth. Lane 7, Sc.EF-Tu1 and EF-Tu3 – S30 supernatant of liquid-grown *S. coelicolor* M145(pISRT3-1); lane 8, EF-Tu2 and Ec.EF-Tu – S30 supernatant of *E. coli* JM101(pUSRT2-3).

product. The Western blot of Fig. 10 revealed the presence of both EF-Tu3 and *S. coelicolor* EF-Tu1 (Sc.EF-Tu1) in the transformed cells (Fig. 10, lane 2) and that of solely

Sc.EF-Tu1 in the parental cells (Fig. 10, lane 1). This demonstrated that Sc.EF-Tu1 is recognized by the antibodies raised against the *tuf-3* product and that it has

the same migration rate as EF-Tu1 of *S. ramocissimus* (Fig. 10, lanes 1, 2 and 4). EF-Tu3 obtained from pISRT3-1 expression in *S. coelicolor* migrated at the same rate as does the product of heterologously expressed *tuf-3*. Therefore we suppose that EF-Tu3, when synthesized in *S. ramocissimus*, behaves similarly during SDS-PAGE. Overexpression of *tuf-2* in *S. coelicolor* has not yet been achieved. But assuming that EF-Tu2 is recognized by both types of antibodies and that it migrates as demonstrated for the product of *tuf-2* expressed in *E. coli*, EF-Tu2 can be detected as well.

Temporal *tuf* gene expression in *S. ramocissimus*

Submerged mycelium of *S. ramocissimus* was cultured for various periods of time in S medium, whereafter the mycelium was sonicated and the resulting suspensions were centrifuged at 30000 g. Under these culture conditions kirromycin is produced. This was demonstrated by extraction of the antibiotic from the medium and analysis with thin-layer chromatography (data not shown). S30 pellets and S30 supernatants were then submitted to SDS-PAGE and immunoblotting using anti-EF-Tu1 (Fig. 11a) and anti-EF-Tu3 (Fig. 11b). EF-Tu1 is clearly present in S30 supernatants (but not in S30 pellets) of exponentially growing cells (20 h cultures, lanes 2 and 3). Larger amounts can be seen, both in the pellet and in the supernatant fractions of cells cultured for 44 h (lanes 4 and 5) and 68 h (lanes 6 and 7). A very faint band is visible at the position of EF-Tu2 but its appearance was not reproducible and degradation of EF-Tu1 cannot be excluded. A signal corresponding to EF-Tu3 was not observed.

Surface-grown cultures of *Streptomyces* show complete morphological differentiation. It was of interest to investigate whether or not expression of *tuf* genes coincides with one or more of the differentiation stages. Surface-grown cells from *S. ramocissimus* were therefore cultured on minimal medium for various periods of time. S30 supernatant fractions, analysed by Western blotting using anti-EF-Tu3 (Fig. 12, lanes 1–6), showed the presence of EF-Tu1 only. A similar analysis of S30 supernatant fractions from pre-germinated spores and aerial hyphae also revealed the presence solely of EF-Tu1 (not shown).

DISCUSSION

The presence of three *tuf*-like genes in the mycelial bacterium *S. ramocissimus* is intriguing. As outlined in the Introduction, the group of Gram-positive bacteria was assumed to harbour only one *tuf* gene. Our results indicate that this is not so in the case of *S. ramocissimus* and most likely not in that of more, if not all, *Streptomyces* spp. (Fig. 4). The location of *tuf-1*, i.e. downstream of *fus*, is similar to that of *E. coli tufA* in the *str* operon and is in line with *tuf-1* encoding a genuine EF-Tu. This is confirmed by the characterization of the *tuf-1* product *in vitro* (Fig. 8). Evidence for a gene organization like that of the *E. coli tufB* operon (four tRNA genes preceding *tufB*) was not obtained for either *tuf-2* or *tuf-3*.

A further striking finding is that the amino acid sequences of EF-Tu1, 2 and 3 from *S. ramocissimus* are not as similar as those of EF-TuA and B from *E. coli*. In the fungus *Rhizomucor racemosus*, the products of the three genes coding for EF-1 α show 99% identity. On the other hand, the eukaryote *Xenopus laevis* has three genes encoding EF-1 α which do not show the strong sequence conservation of their products as observed in other organisms (Djé *et al.*, 1990). Two EF-1 α genes, one expressed in embryos and adult cells (EF-1 α S), the other in oocytes (EF-1 α O), have a high product identity (91%). The EF-1 α (42Sp50) encoded by the third gene has an identity of only 69% compared to the other two (Deschamps *et al.*, 1991). It is found in so-called storage particles as a complex with 5S RNA and various tRNAs (Djé *et al.*, 1990, and references therein). The possibility may be envisaged, therefore, that EF-Tu3 from *S. ramocissimus* has a specialized function. In this context, it is worth mentioning that the identity between EF-Tu1 and EF-Tu2 is 88%, whereas that between EF-Tu3 and the other EF-Tus from the streptomycete is only 65%.

The sequence data do not provide any indication that one of the streptomycete EF-Tus might be kirromycin resistant. Amino acid residues (indicated in bold type in Fig. 3) known to be altered in kirromycin-resistant *E. coli* EF-Tu mutants, i.e. residues 375, 316, 222, 124 (Duisterwinkel *et al.*, 1984; Zeef & Bosch, 1993) and 329 (Abdulkarim *et al.*, 1991) are conserved in all three *S. ramocissimus* EF-Tus. Kirromycin resistance due to residues elsewhere in the molecule cannot be excluded. We, therefore, expressed the three *tuf*-like genes in *E. coli* and studied their temporal expression in *S. ramocissimus*. After heterologous expression of *tuf-1* most of the product can be recovered from *E. coli* in a soluble form. By contrast, heterologous expression of *tuf-2* and *tuf-3* yields inactive products, deposited in inclusion bodies. We do not know what determines the different fate of the three *tuf* products. Incorrect folding of the nascent chain has been invoked to explain this phenomenon, observed after over-expression of foreign genes. If so, it is unclear why the products of *tuf-2* and *tuf-3* should not fold properly during *de novo* synthesis in *E. coli*, whereas the *tuf-1* product folds correctly. Functional differences between the gene products may be considered in this context.

Although functional characterization of only the *tuf-1* gene product has been achieved, heterologous gene expression of all three genes in *E. coli* enabled the identification of each protein and the production of antibodies. A larger difference in molecular mass was observed in the Western blots than expected on the basis of the amino acid sequence derived from the nucleotide sequence (maximally eight amino acid residues). Post-translational modification seems an unlikely explanation since both the *tuf-1* and *tuf-3* products, either homologically or heterologously expressed, displayed the same electrophoretic migration rate.

One of the questions of interest is whether kirromycin production coincides with the expression of one or more of the *tuf* genes. It has been shown in *E. coli* that kirromycin sensitivity is dominant over kirromycin re-

sistance (van de Klundert *et al.*, 1978). Provided that this rule applies to *S. ramocissimus* and that either *tuf-2* or *tuf-3* encodes a kirromycin-resistant EF-Tu, we would expect a clear reduction of the amount of EF-Tu1, which is kirromycin sensitive, and a simultaneous increase in concentration of EF-Tu2 or EF-Tu3. We could only identify unambiguously one *tuf* product, EF-Tu1, in submerged cultures of *S. ramocissimus* under conditions when kirromycin is produced. Surface-grown cultures also produced EF-Tu1 only. If present in *S. ramocissimus*, the intracellular amount of EF-Tu2 and EF-Tu3 is less than a few percent of that of EF-Tu1. These data suggest that protection of *S. ramocissimus* against its own product, kirromycin, is not provided by alteration of EF-Tu. Protection must therefore be achieved by mechanisms such as efficient transport of the antibiotic out of the cell, deposition of it in an intracellular compartment or intracellular modification (for a review see Cundliffe, 1989).

The absence of detectable *tuf-2* and *tuf-3* expression raises the question whether they are silent genes or expressed at very low levels. We opt for the latter and suggest that they have specialized functions not requiring the high concentration normally found for elongation factor Tu. An obvious experimental approach to investigate whether *tuf-2* and/or *tuf-3* are essential would be gene inactivation. Up till now we have been unable to transform *S. ramocissimus*. Other *Streptomyces* spp. such as *S. coelicolor* and *S. lividans* can be transformed readily (Hopwood *et al.*, 1985). Therefore the perspective of studying *tuf-3* inactivation in one of these strains is much more promising.

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