Natural kirromycin resistance of elongation factor Tu from the kirrothricin producer
Streptomyces cinnamoneus

Carmela Cappellano, Federica Monti, Margherita Sosio, Stefano Donadio and Edoardo Sarubbi

Author for correspondence: Stefano Donadio. Tel: +39 2 96474 243. Fax: +39 2 96474 400. e-mail: stefanodonadio@mmd.com

Lepetit Research Center, via R. Lepetit 34, 21040 Gerenzano, Italy

The antibiotic kirromycin (Kr) inhibits bacterial protein synthesis by binding to elongation factor Tu (EF-Tu). Streptomyces cinnamoneus and Nocardia lactamurans, producers of antibiotics of the Kr class, are known to possess an EF-Tu resistant to Kr. Both micro-organisms appear to possess a single tuf gene and we have characterized the one from S. cinnamoneus, which belongs to the tuf1 family. To assess the molecular determinants of Kr resistance, the S. cinnamoneus tuf gene was expressed in Escherichia coli as a translational fusion to maltE, which enabled the recovery by affinity chromatography of the recombinant protein uncontaminated by the host factor. The recombinant EF-Tu was able to catalyse polyU-directed polyPhe synthesis in two heterologous cell-free systems, even as an uncleaved fusion. When tested for antibiotic sensitivity it behaved like the natural S. cinnamoneus protein, showing equivalent resistance to Kr but sensitivity to the antibiotic GE2270, indicating that all determinants for Kr resistance are intrinsic to the EF-Tu sequence. Multiple sequence analysis of EF-Tu proteins, together with knowledge of mutations conferring Kr resistance, allowed the identification of key residues as likely candidates for the natural Kr resistance of the S. cinnamoneus EF-Tu. One of these, Thr278, was mutated to the consensus Ala and the resulting mutant protein was sensitive to Kr. Interestingly, it retained some activity (30% of the control) even at high Kr concentrations.

Keywords: Streptomyces cinnamoneus, antibiotic, expression system, protein synthesis, tuf gene

INTRODUCTION

Elongation factor (EF) Tu is an essential component of bacterial protein synthesis (Weijland et al., 1992). It forms a ternary complex with GTP and aa-tRNA and interacts with the elongating ribosome to place aa-tRNA in the A-site. After codon–anticodon interaction, GTP is hydrolysed, causing the dissociation of EF-Tu*GDP from aa-tRNA and from the ribosome. EF-Tu*GDP is subsequently recycled to an active form via a nucleotide exchange reaction promoted by EF-Ts. The polyenic antibiotics of the kirromycin (Kr) class are known to interact with both EF-Tu*GDP and EF-Tu*GTP, affecting protein synthesis by inhibiting the release of EF-Tu*GDP from the ribosome, thus preventing elongation (Parmeggiani & Swart, 1985). GE2270 (also known as MDL 62879), a structurally unrelated antibiotic, also binds to EF-Tu, but it inhibits protein synthesis through a different mechanism, i.e. by preventing the formation of the aa-tRNA*EF-Tu*GTP complex (Anborgh & Parmeggiani, 1991; Landini et al., 1992).

The protein synthesis machinery of actinomycetes producing antibiotics of the Kr class, although all resistant to these molecules, exhibit different properties (Glöckner & Wolf, 1984). Some producers, such as Streptomyces collinus and Streptomyces ramocissimus, possess a Kr* EF-Tu, with an IC50 of 0.2–0.6 µM. Others, such as Streptomyces cinnamoneus and

Abbreviations: EF, elongation factor; Kr, kirromycin; MBP, maltose binding protein. The EMBL accession number for the nucleotide sequence reported in this paper is X98831.
Nocardia lactamdurans (formerly Streptomyces lactamdurans), possess a Kr factor (IC50 > 500 µM). Different possibilities can be conceived to explain high level resistance in vivo. For instance, a Kr EF-Tu might be replaced with a resistant factor at the onset of antibiotic production. A mechanism of this type, analogous to that found in producers of novobiocin (Thiara & Cundliffe, 1989) and pentanenolactone (Frohlich et al., 1989), would be consistent with the recent finding of multiple tuf genes in Streptomyces (Vijgenboom et al., 1994; van Wezel et al., 1994). Alternatively, an otherwise sensitive target might be made resistant by enzymic modification, a protective mechanism commonly present in producers of protein synthesis inhibitors (Cundliffe, 1989). Finally, the EF-Tu might be intrinsically Kr because of its amino acid sequence. In the latter case, the high conservation of EF-Tu sequences from different organisms and the known mutations conferring Kr (Abdulkarim et al., 1994; Mesters et al., 1994) might provide clues to specific residues implicated in resistance.

S. cinnamoneus produces kirkothricin, an antibiotic of the Kr class presenting only minor structural differences from the latter and presumed to act by a similar mechanism (Thein-Schrammer et al., 1982). In this study we present evidence that S. cinnamoneus possesses a single tuf gene which encodes all the information necessary for a Kr EF-Tu. Remarkably, a similar resistance mechanism also operates in the actinomycete Planobispora rosea, producer of the EF-Tu inhibitor EF-Tu provides clues to the evolution of a resistant target in a producing organism. A preliminary account of this work has been presented (Alderson et al., 1994).

METHODS

Bacterial strains and plasmids. Escherichia coli DH5α was the routine host for cloning, whereas E. coli JM109 was used for gene expression. S. cinnamoneus Tu89 (Thein-Schrammer et al., 1982), Streptomyces coelicolor M145 and Streptomyces lividans 1326 (Hopwood et al., 1985), Streptomyces glaucescens ETH 22794 and N. lactamdurans ATCC 27382 were grown in standard media (Hopwood et al., 1995). E. coli DH5α and Bacillus subtilis ATCC 6633 were used for protein synthesis assays. Plasmids of the pUC series (Vieira & Messing, 1982) and pUCBM21 (Boehringer Mannheim) were used for cloning. pMAL-c2 was from New England Biolabs. Cloning of the S. cinnamoneus tuf gene. For cloning the tuf gene, chromosomal DNA from S. cinnamoneus was digested with BamHI and a 3.5-4.5 kb fragment was recovered from an agarose gel and ligated to BamHI-digested pUCBM21. Plasmid DNA from about 800 of the resulting recombinant colonies was prepared as 100 pools of eight colonies each and analysed by Southern hybridization with the S. coelicolor tuf probe. A single positive pool was identified, from which the single hybridizing plasmid pGE150 was identified.

DNA manipulations. Genomic DNA from actinomycetes was prepared as described by Hopwood et al. (1985). The S. coelicolor tuf segment was amplified using the same conditions used for the P. rosea tuf segment (Sosio et al., 1996), but with an annealing temperature of 50°C. DNA sequences were determined with an ALF automated sequencer (Pharmacia), following the manufacturer’s instructions. Sequence analyses were performed using GCG programs (Devereux et al., 1984). Southern hybridization of genomic DNAs with the S. coelicolor tuf segment was carried out with a hybridization stringency set at 6 x SSC, 65°C, with final washes at 2 x SSC, 65°C (1 x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate). With the S. cinnamoneus tuf probe, the hybridization stringency was set at 6 x SSC, 58°C, with final washes at 2 x SSC at this same temperature.

Plasmid constructions. A 90 bp fragment was PCR-amplified from pGE150, introducing a Smal site at the tuf 5' end and changing the third position in the first few codons. The primers used were 5'TCCCCCGGATGGCAAAAAAATTCCGACCGGAC 3' (bases differing from the S. coelicolor sequence are in italics) and 5' GGTGATCGCCGCGTGAAGATG 3'. This fragment, after digestion with SmaI and SaeII, and the 14 kb Sacl-PstI fragment from pGE150 (Fig. 1; the PstI site originates from the polylinker) were simultaneously ligated into pMAL-c2, previously digested with XmnI and PstI. The resulting plasmid was named pMAL-TUF1. To construct plasmid pMAL-TUF2, the 319 bp NruI-BamHI fragment from pMAL-TUF1 was replaced with the equivalent segment obtained from pGE150 after PCR with primers 5' AGCCGTTCGAGTGGAGGGAAGGCCTTCTGCTTGCCATCC 3' and 5' GACTCTAGAGATCCACAGGTTCTG 3'. The primer changed the ACC codon for Thr 378 into GCC and introduced a silent XmnI site for following the mutant allele. The fidelity of PCR synthesis was verified by DNA sequencing.

Production and purification of recombinant EF-Tu. E. coli JM109 cells harbouring pMAL-TUF1 or pMAL-TUF2 were grown at 30°C in 1 L LB medium containing 50 mg ampicillin ml-1. At an OD600 of 0.4-0.6, IPTG to 0.2 mM and MgCl2 to 10 mM were added. After a further 2 h of growth, cells were harvested and the resulting bacterial pellet was resuspended in 40 ml buffer A (20 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 1 mM DTT and 5 mM GDP) containing 1 mg bovine serum albumin ml-1. Cells were incubated for 30 min in ice, lysed by sonication and then centrifuged to remove cellular debris. The supernatant was diluted fivefold with buffer A and loaded on a 30 ml amylose column (New England Biolabs) pre-equilibrated with the same buffer. Fusion proteins were eluted with buffer A containing 10 mM maltose, concentrated in a speed-vac, dialysed against 20 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 10 mM MgCl2, 1 mM DTT and 5 mM GDP containing 1 mg lysosome ml-1. Cells were incubated for 30 min in ice, lysed by sonication and then centrifuged to remove cellular debris. The supernatant was diluted fivefold with buffer A and loaded on a 30 ml amylose column (New England Biolabs) pre-equilibrated with the same buffer. Fusion proteins were eluted with buffer A containing 10 mM maltose, concentrated in a speed-vac, dialysed against 20 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 10 mM MgCl2, 1 mM DTT and 5 mM GDP containing 1 mg lysozyme ml-1. Cells were incubated for 30 min in ice, lysed by sonication and then centrifuged to remove cellular debris. The supernatant was diluted fivefold with buffer A and loaded on a 30 ml amylose column (New England Biolabs) pre-equilibrated with the same buffer. Fusion proteins were eluted with buffer A containing 10 mM maltose, concentrated in a speed-vac, dialysed against 20 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 10 mM MgCl2, 1 mM DTT and 5 mM GDP containing 1 mg lysozyme ml-1. Cells were incubated for 30 min in ice, lysed by sonication and then centrifuged to remove cellular debris. The supernatant was diluted fivefold with buffer A and loaded on a 30 ml amylose column (New England Biolabs) pre-equilibrated with the same buffer. Fusion proteins were eluted with buffer A containing 10 mM maltose, concentrated in a speed-vac, dialysed against 20 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 10 mM MgCl2, 1 mM DTT and 5 mM GDP containing 1 mg lysozyme ml-1.
Tris/HCl, pH 7.7, 10 mM MgCl₂, 80 mM NH₄Cl, 3 mM DTT, 1 mM GTP, 0.8 mM ATP, containing 80 mg polyU, 0.6 mg E. coli tRNA⁹⁶e, 17 pmol 5-Phe, 3.2 pmol 5-[³⁵S]Phe (2 x 10⁷ Bq mol⁻¹, Amersham), a calibrated amount of S30 extract (to give 10000–20000 d.p.m.) and exogenous EF-Tu when appropriate. Reactions were carried out for 30 min at 30 °C, then stopped by the addition of trichloroacetic acid to 5% (w/v). After heating for 10 min at 80 °C, the precipitate was collected on glass fibre filters using a cell harvester (LKB) and the filter-associated radioactivity measured in a Beta-Plate counter (Pharmacia). To block the endogenous EF-Tu, the S30 extract (to give 10000-20000 d.p.m.) and exogenous EF-Tu when appropriate. Reactions were carried out for 30 min at 30 °C, then stopped by the addition of trichloroacetic acid to 5% (w/v). After heating for 10 min at 80 °C, the precipitate was collected on glass fibre filters using a cell harvester (LKB) and the filter-associated radioactivity measured in a Beta-Plate counter (Pharmacia). To block the endogenous EF-Tu, the S30 extract (to give 10000-20000 d.p.m.) and exogenous EF-Tu were added to the reaction mixture, and the reaction was continued for an additional 30 min. At the end of the reaction, the mixture was cooled on ice, and the precipitate was collected by centrifugation. The filter-associated radioactivity was measured in a Beta-Plate counter (Pharmacia).

Preparation of the S. cinnamoneus S100 fraction. S. cinnamoneus mycelium, grown in 30 g Tryptone Soya 1⁻¹ (Oxoid) for 48 h at 30 °C, was washed with 10% (w/v) sucrose and resuspended in lysis solution (Hopwood et al., 1985) containing 2 mg lysozyme ml⁻¹. After 1 h at 30 °C, the suspension was sonicated on ice, centrifuged for 30 min at 30000 g, and then for 4 h at 100000 g (both spins were at 4 °C). The resulting S100 fraction was mixed with NH₄Cl-washed E. coli ribosomes, prepared as described by Landini et al. (1993), and assayed for polyU-directed polyPhe synthesis, as described above.

RESULTS

Characterization of the S. cinnamoneus tuf gene

A size-enriched library of S. cinnamoneus BamHI fragments (see Methods) was screened by rib selection, probing plasmid DNA isolated from pools of transformants with a tuf fragment. With this procedure, we isolated plasmid pGE150, carrying a 3.8 kb insert, with the tuf gene located within the 1.8 kb NcoI-BamHI fragment (Fig. 1). The nucleotide sequence of this segment revealed an ORF of 1194 nt, encoding a 396 aa polypeptide (after removal of the initial Met) with a calculated Mᵦ of 43760 and pl of 4.96. The translated sequence was closely related (identity scores ranging from 91.7 to 92.2%) to the tuf1 gene products from S. ramocissumus (Vijgenboom et al., 1994), S. coelicolor (van Wezel et al., 1994) and S. collinus (Mikulik & Zhulanova, 1995); lower identity scores (59.9 and 61.0%) were observed with the tuf3 products from the two former species, and with the S. ramocissumus tuf2 product (84.4% identity).

Southern hybridizations employing different digests of each genomic DNA were carried out to establish the number of tuf genes in S. cinnamoneus. When a first experiment was performed, the sequence of the S. coelicolor tuf genes had not been reported. We amplified a tuf fragment from S. coelicolor DNA using consensus oligonucleotides (Sosio et al., 1996) and used this as the probe. A single band was invariably observed with S. cinnamoneus and N. lactamurans DNA, while two bands of approximately equal intensity were observed with S. coelicolor DNA (data not shown). [The size and intensity of these bands were subsequently found to correspond to S. coelicolor tuf1 and tuf3 (van Wezel et al., 1994).] We then used the Smal-Nrul fragment from the S. cinnamoneus gene (Fig. 1) as the probe. Under moderate stringency, we could detect a strong and a weak band in each digest of S. coelicolor DNA, corresponding in size to tuf1 and tuf3, respectively. A similar hybridization profile (a strong and a weak band) was also observed with S. glaucescens and S. lividans DNA. However, only a single strong band was detected with S. cinnamoneus and N. lactamurans DNA (data not shown). The results of these hybridizations strongly suggest that the latter two species possess a single tuf gene.

A GC content of 63.6% for the S. cinnamoneus tuf gene is a relatively low but not unprecedented value for Streptomyces genes (Wright & Bibb, 1992). The frequent use of some T-ending codons (e.g., GGT and CGT account for 44 and 55% of the Gly and Arg codons, respectively) observed in tuf is believed to be characteristic of highly expressed genes in high GC microorganisms (Ohama et al., 1990; Wright & Bibb, 1992). Upstream to tuf we found the last 62 codons of an ORF highly related to fus, encoding EF-G. Downstream, 39 bp after the stop codon TAA, there is a putative transcription terminator (a 15 bp inverted repeat followed by six Ts), with a calculated ΔG of −47 kcal mol⁻¹ (Tinoco et al., 1973) for the corresponding RNA. The fus–tuf organization typical of tuf1 loci (Sosio et al., 1996; van Wezel et al., 1994; Vijgenboom et al., 1994) and the high relatedness to known tuf1 genes indicate that the single S. cinnamoneus tuf gene belongs to the tuf1 class.

Interestingly, the segment immediately preceding the tuf GTG start codon was found to be highly conserved among the four Streptomyces tuf1 sequences available (Fig. 2). With the accommodation of a few gaps, there are 52/64 invariant positions among the four species. The extent of conservation declines considerably further upstream from this conserved segment. Conversely, no significant similarity among the four species was
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positions were identified in this way (Fig. 2) from the consensus in highly conserved positions. Four positions have been mapped on the EF-Tu sequence (Abdulkarim et al., 1994). Additional deviations from the consensus in the S. cinnamoneus sequence are at positions 278 (Ala instead of Val/Ile/Thr). The major deviations from the consensus in the S. cinnamoneus sequence are marked by arrows. Abbreviations are defined in the legend to Fig. 2.

Fig. 2. Alignment of the region upstream to tuf1 in S. collinus (Scol), S. ramossstimus (Sram), S. coelicolor (Scoe) and S. cinnamoneus (Scin). The unmarked bottom line indicates the deduced consensus sequence. The translational start site is taken as position +1.

Fig. 3. Alignment of selected portions of Streptomyces EF-Tu sequences. Only positions different from the S. coelicolor EF-Tu (Scoe) are indicated. The unmarked bottom line indicates the consensus sequence (18 out of 22 positions) calculated using the programsPILEUP and PRETTY, using a set of aligned EF-Tu proteins (see Sosio et al., 1996, for details). The major deviations from the consensus in the S. cinnamoneus sequence are marked by arrows. Abbreviations are defined in the legend to Fig. 2.

detected 3’ to tuf. A putative ribosome binding site can be recognized in this conserved segment. However, no sequence clearly resembling any from a compilation of Streptomyces promoters (Strohl, 1992) could be detected. These observations suggest an important, although still unknown, function for the region preceding tuf1 in Streptomyces.

Analysis of the deduced EF-Tu sequence

Several single amino acid substitutions conferring KrR to E. coli have been mapped on the EF-Tu sequence (Abdulkarim et al., 1994; Mesters et al., 1994). We thus checked the S. cinnamoneus EF-Tu for substitutions equivalent to those found in resistant mutants, as well as for deviations from the consensus in highly conserved positions. Four positions were identified in this way (Fig. 3). The most striking substitutions are found at position 127 (corresponding to 125 in E. coli) with a Ser in place of the invariant Val, and at position 378 (375 in E. coli) where a Thr substitutes the consensus Ala. This latter position is particularly intriguing, considering that the A375T mutation confers Krr to E. coli and Salmonella typhimurium (Parmeggiani & Swart, 1985; Abdulkarim et al., 1994; Mesters et al., 1994). Additional deviations from consensus are at positions 294 (Cys in place of the invariant Val) and 359 (Ala instead of Val/Ile/Thr).

The availability of three highly related sequences from KrR Streptomyces EF-Tu proteins enabled a further inspection of amino acid residues peculiar to the S. cinnamoneus protein. The four sequences contain 327 identical residues out of 397. Only at 27 positions is one sequence different from the other three: the S. cinnamoneus EF-Tu has 18 unique residues, whereas the other three proteins differ at 1, 3 or 5 positions (Fig. 3).
Interestingly, half of these unique residues are found in the 291–397 segment of the \textit{S. cinnamoneus} EF-Tu.

**Expression in \textit{E. coli}**

If the above-described deviations from consensus are sufficient \textit{per se} to confer \textit{Kr}^\text{R}, then expression of the \textit{S. cinnamoneus} \textit{tuf} gene in a heterologous host such as \textit{E. coli} should produce a resistant EF-Tu. However, any contamination of the recombinant protein by the endogenous \textit{Kr}^\text{R} EF-Tu must be avoided, since this would interfere in protein synthesis assays, due to the dominance of \textit{Kr}^\text{R} \cite{Parmeggiani & Swart, 1985}. To achieve this separation easily, it was necessary to add an affinity tag to the recombinant protein. We tried the C-terminal hexaHis tag expression system \cite{Boon et al., 1992}; however, under the same conditions in which some soluble \textit{E. coli} EF-Tu was obtained, the His-tagged \textit{S. cinnamoneus} protein was completely insoluble \cite{Donadio, unpublished results}. As an alternative method, we fused the \textit{tuf} gene to the 3' end of \textit{malE}. The resulting fusion protein could be purified easily by affinity chromatography and subsequently released from the fusion partner by exploiting a Factor Xa cleavage site located in the linker region between the two moieties. The final EF-Tu product would contain, at its N terminus, one (Gly) or two (if Met is cleaved off in the natural protein) additional residues. SDS-PAGE analysis showed an IPTG-inducible, amylase-binding protein of the expected 85 kDa size which, after cleavage by Factor Xa, yielded two proteins of 47 and 40 kDa. These were identified as EF-Tu and MBP, respectively, by Western blotting with anti-EF-Tu and anti-MBP antibodies (data not shown). The intact fusion protein or the EF-Tu obtained upon cleavage contained a functional GDP-binding domain, as indicated by their ability to bind GDP-Sepharose.

The recombinant EF-Tu was tested for its ability to catalyse protein synthesis in cell-free systems. We found that addition of the \textit{S. cinnamoneus} protein to S30 extracts from \textit{E. coli} or \textit{B. subtilis} resulted, in both cases, in a marked increase in polyU-directed polyPhe synthesis. A similar effect was shown by the uncleaved fusion and by \textit{E. coli} EF-Tu, used as a positive control. This finding indicated that in both S30 extracts the amount of active endogenous EF-Tu present was limiting and that the \textit{S. cinnamoneus} protein was functional.

To confirm this finding and to obtain quantitative data on the functionality of the recombinant protein, the endogenous EF-Tu in both S30 extracts was specifically inhibited by adding a calibrated amount of GE2270, which inactivates EF-Tu, but not ribosome function \cite{Anborgh & Parmeggiani, 1991; Landini et al., 1992}. After inhibition of the endogenous factor, the ability of exogenously added EF-Tu to restore protein synthesis was evaluated by comparing the \textit{S. cinnamoneus} protein and the similarly produced \textit{P. rosea} EF-Tu \cite{Sosio et al., 1996} with the natural \textit{E. coli} EF-Tu. The \textit{S. cinnamoneus} protein restored full protein synthesis activity in both systems, both as an uncleaved fusion or as a cleaved product. Fig. 4 shows that similar amounts of \textit{S. cinnamoneus} EF-Tu were required to restore 50% of the original polyU-directed polyPhe synthesis activity to GE2270-inactivated S30 extracts from (a) \textit{E. coli} and (b) \textit{B. subtilis}. The respective activities before GE2270 addition were 13800 and 12600 c.p.m. Factor Xa-cleaved and uncleaved fusion proteins are indicated by white and grey bars, respectively. Natural \textit{E. coli} EF-Tu is indicated by the black bars. T378A denotes the \textit{S. cinnamoneus} mutant EF-Tu.

![Fig. 4. Activity of recombinant EF-Tu proteins in protein synthesis. The bars denote the amount of protein required to restore 50% of the original polyU-directed polyPhe synthesis activity to GE2270-inactivated S30 extracts from (a) \textit{E. coli} and (b) \textit{B. subtilis}. The respective activities before GE2270 addition were 13800 and 12600 c.p.m. Factor Xa-cleaved and uncleaved fusion proteins are indicated by white and grey bars, respectively. Natural \textit{E. coli} EF-Tu is indicated by the black bars. T378A denotes the \textit{S. cinnamoneus} mutant EF-Tu.](image)

The lower activity exhibited by the recombinant EF-Tu in comparison to the natural \textit{E. coli} factor could be due to the additional residue(s) at the N terminus of the cleaved product. Protein preparations active in cell-free protein synthesis were obtained only after growing the \textit{E. coli} host cells at 30 °C; higher temperatures resulted in totally inactive products. Nonetheless, our results show that N-terminal MBP fusions can represent a useful method to produce an EF-Tu easily resolvable from the endogenous factor and sufficiently active for characterization of its antibiotic resistance.

**Resistance to \textit{Kr}**

Before evaluating the behaviour of the recombinant EF-Tu towards \textit{Kr}, its natural counterpart was assayed. To this end, an S100 fraction (containing EF-Tu and all other soluble factors) from \textit{S. cinnamoneus} was combined with \textit{E. coli} ribosomes and tested in polyU-directed polyPhe synthesis. This system was highly resistant to \textit{Kr}, with only 40% inhibition observed at concentrations as high as 100 μg ml$^{-1}$, in agreement with previous reports \cite{Glöckner & Wolf, 1984}. As expected,
mapped in other EF-Tu proteins (Parmeggiani et al., 1993).

As described above, Thr378 in the Kr' S. cinnamoneus tuf gene, derived as the natural protein with respect to antibiotic activity, shows a different response to Kr (even at high Kr concentrations (Fig. Sb)). Conversely, EF-Tu was only marginally affected by Kr (even at high Kr concentrations (Fig. Sb)). The response to Kr, in contrast, was very different between the two proteins: the T378A mutant EF-Tu behaved essentially as a Kr' protein (Fig. Sa), with most of the activity being lost at less than 1 µg Kr ml⁻¹. However, considerable residual activity (30% of the control) was retained even at 100 µg Kr ml⁻¹, in contrast with the Kr' E. coli EF-Tu. When the experiment was performed with the GE2270-inhibited extract, the T378A protein again showed an apparently biphasic inhibition by Kr, with over 30% residual activity at 100 µg Kr ml⁻¹ (Fig. 5b). This result makes it unlikely that the observed residual activity was due to the endogenous Kr' B. subtilis EF-Tu.

In conclusion, these results indicate that the Thr378 residue as an important determinant of Kr' in the naturally resistant S. cinnamoneus protein. They also suggest that other residues in the EF-Tu primary structure may contribute to resistance.

**DISCUSSION**

By expressing the tuf gene in E. coli and characterizing the resulting product, we have found that all the information required for the synthesis of a Kr' EF-Tu is contained within the single S. cinnamoneus tuf gene. The importance of Thr378 in conferring Kr' to the S. cinnamoneus EF-Tu, suggested by the gene sequence, was verified experimentally by characterizing the T378A mutant, which behaved essentially as a Kr' factor. However, this mutant retained some residual protein synthesis activity at high Kr concentrations. This may suggest either a low affinity for the antibiotic, or alternatively that the mutant EF-Tu*Kr complex is somehow able to catalyse protein synthesis, although at much lower efficiency. The fact that the mutant activity decreased by 20% at 0.1 µg Kr ml⁻¹ and that it did not change significantly between 1 and 100 µg Kr ml⁻¹ (Fig. 5a), argues against the former hypothesis. In any case, the residual activity of the T378A mutant strongly suggests that some other amino acid residue is involved in conferring protection from the antibiotic.

Most of the mutations conferring Kr' to E. coli and S. typhimurium factors, when superimposed on the three-dimensional structure of the Thermus thermophilus EF-Tu complexed with a GTP analogue (Berchtold et al., 1993), map at the interface region between domains I and III. It has been postulated that Kr binds to this region, possibly at the interface itself (Mesters et al., 1994). Consistent with this hypothesis is the finding that many of the amino acid residues unique
to the *S. cinnamoneus* sequence, when compared to the other *Streptomyces* EF-Tu proteins, occur within domain III, particularly in the C-terminal 50 aa segment (Fig. 3). Among the deviations from consensus seen in the *S. cinnamoneus* EF-Tu, Ser^157^ (in place of the invariant Val) is particularly interesting. To our knowledge, this position has never been implicated in Kr^r^ before. Nevertheless, it is located at the domain I-III interface, adjacent to Gln^158^ (*E. coli* numbering), a residue that when mutated to Lys confers high level Kr^r^ (Zeef & Bosch, 1993). In addition, Ser^157^ is spatially very close to Thr^276^ in the GTP-bound structure of the *T. thermophilus* EF-Tu (Berchtold et al., 1993).

Most *Streptomyces* species possess multiple *tuf* genes, and a *tuf3*-like gene appears to be present in each case (van Wezel, 1994). For example, *S. ramocissimus*, a Kr producer with a Kr^r^ EF-Tu, has three such genes (Vijgenboom et al., 1994). We failed to detect a *tuf3* homologue in *S. cinnamoneus*, however, although we cannot exclude the presence of a very divergent *tuf3*. In *E. coli*, due to the dominance of Kr^r^, resistance can be usually selected in the presence of a null mutation in *tufB* (Parmeggiani & Swart, 1985). It is tempting to speculate that acquiring a Kr^r^ EF-Tu in *Streptomyces* might require loss of the extra *tuf* genes, although *tuf2* and *tuf3* expression in *S. ramocissimus* has yet to be observed (Vijgenboom et al., 1994). [The only indication of *tuf3* expression, restricted to a very narrow growth range, comes from *S. coelicolor* (van Wezel et al., 1995).]

The existence of some Kr producers with a sensitive EF-Tu (Glöckner & Wolf, 1984) suggests the existence of additional resistance determinant(s), likely to be linked to the Kr biosynthesis genes, as found in other antibiotic producing actinomycetes (Martin & Liras, 1989). These additional protective mechanisms may allow the evolution of a resistant target which is as functional as the sensitive one. This has probably occurred through multiple mutations in the *tuf* gene, as suggested by the observation that the Kr^r^ *S. cinnamoneus* EF-Tu has diverged somewhat from the other streptomycete *tuf1* gene products, particularly in domain III. This hypothesis is consistent with the finding that most mutants selected for Kr^r^ appear to have an EF-Tu less active than its wild-type counterpart (Abdulkarim et al., 1994). The need for multiple substitutions to reconcile antibiotic resistance with full functionality has been recently reported for the *rpsL* gene involved in conferring streptomycin resistance (Schrag & Perrot, 1996).

In conclusion, a resistant EF-Tu from an antibiotic producer can offer valuable insights not only into the molecular determinants of antibiotic resistance, but also into specific interactions important for normal functioning of this essential protein synthesis factor.

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