Characterization of *Mycobacterium tuberculosis* ribosome recycling factor (RRF) and a mutant lacking six amino acids from the C-terminal end reveals that the C-terminal residues are important for its occupancy on the ribosome

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**INTRODUCTION**

*Mycobacterium tuberculosis* is responsible for more casualties worldwide than any other infectious agent. The reemergence of tuberculosis as a serious infection, especially in AIDS patients, and the availability of the complete genome sequence of *M. tuberculosis* have been the major driving forces behind much of the recent research on this organism (Clark-Curtis, 1990; Young & Cole, 1993; Kaufmann & van Embden, 1993; McFadden, 1996; Cole et al., 1998). The structural and functional analyses of the essential gene products associated with tubercle bacilli constitute a crucial component in the discovery of new drug targets to control the growth of *M. tuberculosis*.

Ribosome recycling factor (RRF) is an essential factor for protein synthesis in bacteria and in eukaryotic organelles (Janosi et al., 1996; Kaji et al., 1998). RRF is required for the disassembly of the post-termination complex. Interestingly, in eukaryotes RRF is only needed...
in the organelles, making it a novel drug target (Janosi et al., 1996; Kaji et al., 1998). Also, in Staphylococcus aureus the levels of RRF increase upon infection of animal cells (Lowe et al., 1998). Furthermore, in brucellosis (caused by Brucella melitensis) the sera from infected sheep show antibodies against RRF, suggesting this protein to be a virulence factor (Vizcaino et al., 1996). Thus, RRF may even be of interest in developing subcellular vaccines. The RRFs from different bacteria are highly conserved in their primary structure (Fig. 1), and the three-dimensional structure of the RRFs from four organisms (Thermotoga maritima, Thermus thermophilus, Escherichia coli and Aquifex aeolicus) are known (Selmer et al., 1999; Toyada et al., 2000; Kim et al., 2000; Yoshida et al., 2001). These structures show that the overall architecture of the different RRFs, consisting of two domains, is also highly conserved and mimics tRNAs in its size and shape. Domain I is represented by three long α-helices and domain II is composed of a β-α-β sandwich. The two domains are connected to each other by two loops. Domains I and II represent the long and short arms, respectively, of the ‘L’-shaped tRNA.

In spite of the remarkable structural similarity between the different RRFs, cross-genus-complementation experiments show that while the RRF from Pseudomonas aeruginosa functions efficiently in E. coli, the RRF from T. thermophilus (TthRRF) does not (Ohnishi et al., 1999; Fujiwara et al., 1999). The reasons for this functional difference are not understood. Interestingly, a mutant of TthRRF lacking five residues from the C-terminal end complemented an E. coli strain defective in its frr gene (Fujiwara et al., 1999). The creation of similar mutations in E. coli RRF (EcoRRF) showed that mutants which lacked up to seven residues from the C-terminal end were still functional. However, deletions that resulted in the loss of nine or more residues from the C-terminal end were defective in ribosome recycling (Fujiwara et al., 2001; Janosi et al., 2000).

Recently, we have shown that, like TthRRF, M. tuberculosis RRF (MtuRRF) also failed to complement an frrΔ6 strain of E. coli LJ14. However, simultaneous expression of the elongation factor G (EFG) and the RRF from M. tuberculosis resulted in the rescue of the temperature-sensitive phenotype of the LJ14 strain, highlighting the importance of specific interactions between the two proteins (Rao & Varshney, 2001). To understand the mechanism of action of MtuRRF further, in this study we have generated a mutant of MtuRRF that lacks the last six amino acids of the C-terminal end (equivalent to ΔC5 of T. thermophilus), investigated its biochemical and biophysical properties and carried out functional analyses in E. coli.

METHODS

E. coli strains and growth conditions. E. coli strains (Table 1) were grown in Luria–Bertani (LB) liquid or solid (with 1.5% agar) medium (Sambrook et al., 1989), unless stated otherwise. The medium was supplemented with tetracycline (12.5 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) as required. When needed, IPTG was added to the medium to a final concentration of 0.5 mM. For the growth rate experiments, cultures were started with a 0.6% (v/v) inoculum from freshly grown (at 30 °C) overnight cultures, and the growth at both the permissive (30 °C) and non-permissive (42 °C) temperatures was monitored by recording culture densities at 600 nm at regular intervals.

Cloning of RRF and EFG from M. tuberculosis and E. coli. The ORFs of the genes were cloned into vectors pTrc99C, pET11d and pACDH (Rao & Varshney, 2001).

Construction of ΔC6MtuRRF. The mutant lacking six amino acids from the C-terminal end of MtuRRF (ΔC6MtuRRF) was generated by PCR from pTrcMtuRRF. PCR was carried out with Pfu DNA polymerase as described previously for MtuRRF (Rao & Varshney, 2001) using a forward primer (5'-GGGCCCCATGGTTAGGAGCTCTCTC-3') containing an NcoI site and a reverse primer (5'-AGCAAGCTTATTCTTTGTTAACC-3') which incorporated a stop codon at position 180 (G to amber) and a HindIII site. The
PCR product was digested with NcoI and HindIII and cloned into the respective sites of the CoE1 origin-of-replication-based vectors pTrc99C and pET11d to generate pTrcAC6MtuRRF and pETAC6MtuRRF, respectively. The same fragment was also subcloned into pACDH containing a pACYC origin of replication to yield pACDHAC6MtuRRF. Incorporation of the fragment was confirmed by complete DNA sequencing.

**Purification of MtuRRF and ΔC6MtuRRF, N-terminal sequencing and electron spray ionization-mass spectroscopy (ESI-MS).** MtuRRF and ΔC6MtuRRF were purified from *E. coli* BL21(DE3) harbouring either pETMtuRRF or pETAC6MtuRRF, respectively. The transformants were inoculated into 2xYT (Sambrook et al., 1989) (2 l) and induced with 0.5 mM IPTG at the mid-exponential phase of growth (OD$_{600}$ value of between 0.3 and 0.4). Cells were harvested, sonicated and used to obtain the S100 lysate. The S100 lysate was subjected to streptomycin sulfate (0.9% precipitation), and the supernatant was subjected to ammonium sulfate precipitation (90% saturation). The precipitate was recovered by centrifugation, dissolved in 1 ml of 20 mM Tris/HCl (pH 7.4) and dialysed against the same buffer for 12 h. Dialysed sample was loaded onto a Superdex 75 column (Amersham Pharmacia Biotech). Proteins were eluted with 20 mM Tris/HCl (pH 7.4), 500 mM NaCl and 10% (v/v) glycerol, and the fractions enriched for RRF (as analysed by SDS-PAGE) were pooled, made lyophilized against 20 mM Tris/HCl (pH 7.4) and subjected to Mono Q column chromatography. The proteins were eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris/HCl (pH 7.4) and 10% glycerol buffer. The fractions enriched for RRF were dialysed against 20 mM HEPES (pH 5.0) and loaded onto a hydroxyapatite column (Bio-Rad); the RRF was eluted using 20 mM HEPES (pH 5.0), 200 mM NaCl and 10% glycerol. The protein microsequence and ESI-MS analyses were carried out by the respective facilities at the Indian Institute of Science, Bangalore, India.

**Purification of *E. coli* and *M. tuberculosis* EFGs.** EFGs were purified from *E. coli* BL21(DE3) using the T7 RNA polymerase expression constructs pETMtuEFG and pETEcoEFG (Rao & Varshney, 2001).

**Gel electrophoresis.** Proteins were electrophoresed on 15 and 12% polyacrylamide gels containing 0.1% SDS (SDS-PAGE) and visualized by Coomassie brilliant blue R-250 staining (Laemmli, 1970). The non-denaturing PAGE (native PAGE) was performed in the same way but lacked SDS. Loading dye for native gels consisted of 50 mM Tris/HCl (pH 6.8), 10% (v/v) glycerol and 0.01% bromophenol blue.

**Circular dichroism (CD) spectroscopy.** CD measurements were done on an automated JASCO-J715 spectropolarimeter using 0.2 cm and 1 cm path length quartz cuvettes for secondary and tertiary CD spectra, respectively. Samples were prepared in 20 mM potassium phosphate buffer (pH 7.0). Each spectrum was a mean of four scans with a slit width of 1 nm, response time of 4 s and a scan speed of 50 nm s$^{-1}$.

**Polysome preparation, in vitro ribosome recycling and polysome binding assays.** Polysomes were prepared from *E. coli* MRE600 and used in ribosome recycling assays (Tai & Davis, 1979; Girbes et al., 1979; Rao & Varshney, 2001). To perform binding assays, factor-free polysomes (OD$_{245}$ ~ 2) were incubated in reaction volumes of 250 µl without or with RRFs in the RRF assay buffer (10 mM Tris/HCl, pH 7.4, 80 mM NH$_4$Cl, 8.2 mM MgSO$_4$, 1 mM DTT, 10 µM puromycin, 160 µM GTP) at 33 °C for 20 min and the reaction mixture was layered on a mini-column (1 ml) packed with Sepharose 4B matrix and centrifuged at 2500 r.p.m. for 3 min in a table-top centrifuge. The eluate was concentrated by vacuum drying, separated by SDS-PAGE (15%) and analysed by immunoblotting (Towbin et al., 1979).

**Immunoblotting.** The cell-free extracts (10 µg total proteins) or the ribosome samples (in the RRF binding studies) were separated by SDS-PAGE (12%) and electroblotted onto a PVDF membrane (Towbin et al., 1979) at 200 mA for 2 h. The blots were probed with anti-MtuRRF rabbit antibodies and detected using alkaline phosphatase-conjugated goat-anti-rabbit IgG with the substrates p-nitroterazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate.

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**Table 1. List of *E. coli* strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant details*</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Strain</td>
<td></td>
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<tr>
<td>L14</td>
<td>MC1061 containing frr14* allele</td>
<td>Janosi et al. (1998)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (∆Iets857 ind1 sam7 min5 lacUV5–T7 gene 1)</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pACDH</td>
<td>Harbours ACYC origin of replication; derived from pACD (Mangroo et al., 1995) by mutating the HindIII site of the Tet* marker to the Nbel site</td>
<td>T. K. DineshKumar &amp; U. Varshney (unpublished data)</td>
</tr>
<tr>
<td>pACDHAC6MtuRRF</td>
<td>M. tuberculosis RRF cloned into pACDH</td>
<td>Rao &amp; Varshney (2001)</td>
</tr>
<tr>
<td>pACDHAC6MtuRRF</td>
<td>M. tuberculosis RRF (∆C6 mutant) cloned into pACDH</td>
<td>This work</td>
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<tr>
<td>pTrcEcoEFG</td>
<td>E. coli EFG cloned into pTrc99C</td>
<td>Rao &amp; Varshney (2001)</td>
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<tr>
<td>pTrcMtuEFG</td>
<td>M. tuberculosis EFG cloned into pTrc99C</td>
<td>Rao &amp; Varshney (2001)</td>
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<tr>
<td>pETAC6MtuRRF</td>
<td>pET11d-based overexpression construct of ΔC6MtuRRF</td>
<td>This work</td>
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<td>pET11d-based overexpression construct of MtuEFG</td>
<td>Rao &amp; Varshney (2001)</td>
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* Tet*, tetracycline-resistant.
RESULTS

Purification of RRF

*Mtu*RRF (~21 kDa) was purified to apparent homogeneity by chromatography on Superdex 75, MonoQ and hydroxyapatite columns (Fig. 2a). Microsequencing of this preparation yielded an N-terminal sequence, ?DEALFDAEE..., which matched with the N-terminal of *Mtu*RRF sequence deduced from its gene, suggesting that the initiator fMet was cleaved from the mature protein. The ESI-MS analysis also showed a major peak of 20720 Da that corresponded to the protein lacking the N-terminal fMet. However, in this analysis, a minor peak of 20851 Da corresponding to a polypeptide in which fMet had not been removed was also seen. This was most likely due to hyperexpression of *Mtu*RRF.

![Fig. 2. Purification and characterization of RRFs. (a) Purification profile of *Mtu*RRF on SDS-PAGE (15%). Lanes: 1 and 2, aliquots (~8 µg) from S20 and S100 extracts, respectively; 3–5, aliquot (~4 µg) from the pool of eluates from Superdex 75, Mono Q and hydroxyapatite columns, respectively. (b) Analysis of purified RRFs (~4 µg each protein) on SDS-PAGE (15%). Lanes: 1, molecular mass marker (Sigma); 2, ΔC6*Mtu*RRF; 3, EcoRRF; 4, *Mtu*RRF. (c) Analysis of purified RRFs on 15% PAGE (native). Lanes: 1, ΔC6*Mtu*RRF (~2 µg); 2, EcoRRF (~2 µg); 3, *Mtu*RRF (~3 µg).]

![Fig. 3. CD spectroscopy. (a) Secondary structure and (b) tertiary structure CD spectra of *Mtu*RRF (●, a; ○, b) and ΔC6*Mtu*RRF (○, a; ●, b).]

![Fig. 4. Binding of *Mtu*RRF and ΔC6*Mtu*RRF to E. coli polysomes. The binding to polysomes was analysed using a spin-column method (see Methods). Lanes: 1, ΔC6*Mtu*RRF alone (5 µg); 2, ΔC6*Mtu*RRF alone (50 µg); 3, *Mtu*RRF (5 µg) and ΔC6*Mtu*RRF (5 µg); 4, *Mtu*RRF (5 µg) and ΔC6*Mtu*RRF (25 µg); 5, *Mtu*RRF (5 µg) and ΔC6*Mtu*RRF (50 µg); 6, *Mtu*RRF (5 µg); 7, polysomes alone; 8, *Mtu*RRF (5 µg) and ΔC6*Mtu*RRF (50 µg) without polysomes; M1 and M2, marker lanes for *Mtu*RRF and ΔC6*Mtu*RRF, respectively.]

ΔC6MtuRRF and EcoRRF were also purified to apparent homogeneity using the methodology described above. The comparative analysis of the full-length RRFs (MtuRRF and EcoRRF) by SDS-PAGE showed that in spite of their similar sizes (185 aa each) and closely related molecular masses, MtuRRF (Fig. 2b, lane 4) migrated somewhat slower than EcoRRF (Fig. 2b, lane 3). As expected, ΔC6MtuRRF migrated slightly faster than the wild-type form, MtuRRF (Fig. 2b, compare lanes 2 and 4). However, the migration of the three RRFs on native PAGE (Fig. 2c) was according to their calculated pI values (6.16, 6.45 and 5.71 for ΔC6MtuRRF, EcoRRF and MtuRRF, respectively). Since all three of the RRFs had similar molecular masses (20–18, 20–84 and 20–82 kDa, respectively), the observed mobility profile on the native gel implied that the overall architecture of these proteins was likely to be similar.

CD spectroscopy

The X-ray structures of RRFs have shown that the two domains that mimic the arms of the ‘L’-shaped tRNA consist of well-defined secondary structural elements (Yoshida et al., 2001; Selmer et al., 1999; Kim et al., 2000; Toyoda et al., 2000). Domain I consists of three long α-helices. This property of RRF makes it a suitable molecule for CD spectroscopic analysis to probe for any major structural changes that could result from the introduction of mutations. As seen in Fig. 3(a), the secondary CD profiles of the wild-type (MtuRRF) and the mutant (ΔC6MtuRRF) proteins are almost identical, suggesting that the deletion of six residues from the C-terminal end of MtuRRF does not result in its inappropriate folding. Furthermore, although the tertiary CD signals are weak (MtuRRF lacks tryptophans), the similar spectra for the two proteins (Fig. 3b) suggest that even the overall architecture of the two proteins is similar.

Polysome binding assays

We carried out polysome binding assays by a novel spin-column method using Sepharose 4B matrix, which permitted elution of ribosome-bound RRF but not free RRF into the excluded volume (Fig. 4). This analysis showed that although ΔC6MtuRRF and MtuRRF bound to ribosomes (Fig. 4, lanes 1 and 6, respectively), the binding of ΔC6MtuRRF was weaker than that of MtuRRF. However, when ΔC6MtuRRF was taken in a large amount (50 μg), its binding to ribosomes was comparable to that of the full-length protein (5 μg) (Fig. 4, compare lanes 2 and 6). And, as expected, when the free polysomes, or the same amounts of free RRFs, were spun through the mini-columns, no signals corresponding to RRFs were seen (Fig. 4, lanes 7 and 8, respectively).

Fig. 5. Analysis of complementation of E. coli L14 (frt) with ΔC6MtuRRF. (a) Various plasmids were introduced into E. coli L14 and the fresh cultures of the transformants (uninduced or induced with 0.5 mM IPTG) were streaked onto LB agar plates containing ampicillin and tetracycline with or without IPTG, respectively, in duplicate. One plate was incubated at the permissive temperature (30 °C) and the other at the non-permissive temperature (42 °C). Sectors: 1, pACDH and pTrc99C; 2, pACDHΔC6MtuRRF and pTrc99C; 3, pACDHΔC6MtuRRF and pTrcMtuEFG; 4, pACDHΔC6MtuRRF and pTrcEcoEFG; 5, pACDHΔMtuRRF and pTrcMtuEFG. (b) Growth of E. coli L14 (frt) transformants at the permissive (30 °C) and non-permissive (42 °C) temperatures. Cultures in LB containing ampicillin, tetracycline and 0.5 mM IPTG were started with 0.06% inoculums from overnight cultures (30 °C) and were grown at the permissive (30 °C, solid symbols) and non-permissive (42 °C, open symbols) temperatures. The growth of the cultures (OD₆₀₀) was monitored at regular intervals. The transformants harboured either vectors alone (pACDH and pTrc99C) or various recombinants expressing RRF and EFG, as indicated in the inset. (c) Detection of ΔC6MtuRRF expression in E. coli L14 by immunoblotting using anti-MtuRRF antibodies. Protein extracts (10 μg total protein) prepared from cultures induced (+) with 0.5 mM IPTG or uninduced (−) cultures were separated by SDS-PAGE, transferred to a PVDF membrane and then probed with anti-MtuRRF antibodies. Details of the various plasmids in the transformants are as shown.
Furthermore, we carried out competitive binding analyses of the two RRFs to polysomes (Fig. 4, lanes 3–5). As seen from the band intensities corresponding to the two proteins, in the presence of a fixed amount of MtuRRF (5 µg) and increasing amounts of ΔC6MtuRRF (5 to 50 µg), the binding of the latter to ribosomes increased with a concomitant decrease in the binding of MtuRRF. Consistent with the analyses in Figs 2(b) and 3(a, b), the ability of ΔC6MtuRRF to bind to the ribosomes further supported the view that it folded correctly.

Complementation analysis of *E. coli* LJ14 with ΔC6MtuRRF

As the deletion of the C-terminal five amino acids from *Tth*RRF bestowed upon it the ability to complement an frr<sup>sa</sup> strain of *E. coli*, it was of interest to us to investigate the phenotype of an equivalent mutant of *Mtu*RRF. However, our analysis showed that ΔC6MtuRRF failed to rescue the temperature-sensitive phenotype of *E. coli* LJ14 (frr<sup>sa</sup>) even upon its induction with IPTG (Fig. 5a, sector 2, compare growth at the permissive temperature to growth at the non-permissive temperature). Simultaneous overproduction of *E. coli* EFG (EcoEFG) also did not confer the ability to ΔC6MtuRRF to rescue the temperature-sensitive phenotype of *E. coli* LJ14 at the non-permissive temperature (Fig. 5a, sector 3). In fact, ΔC6MtuRRF failed to complement *E. coli* LJ14 even when co-expressed with *M. tuberculosis* EFG (MtuEFG) (Fig. 5a, sector 4). However, as reported earlier (Rao & Varshney, 2001), the wild-type *Mtu*RRF complemented *E. coli* LJ14 in the presence of MtuEFG (Fig. 5a, sector 5) irrespective of induction with IPTG. To further verify these results, we monitored the growth of the transformants in liquid cultures (Fig. 5b). Consistent with the plating experiment, the transformants harbouring the vectors or the ΔC6MtuRRF constructs alone or along with the plasmids harbouring EcoEFG or MtuEFG grew at the permissive temperature but not the non-permissive temperature. The immunoblot analysis of the cellular extracts of the transformants (grown at the permissive temperature) showed that ΔC6MtuRRF was produced in *E. coli* LJ14 (Fig. 5c, lanes 1–6) and its expression increased upon induction with IPTG (Fig. 5c, compare lanes 1, 3 and 5 with 2, 4 and 6). The levels of ΔC6MtuRRF produced upon induction with IPTG (Fig. 5c, lanes 2, 4 and 6) were comparable to those of wild-type *Mtu*RRF in uninduced cultures (Fig. 5c, lane 7), suggesting that the failure of complementation of *E. coli* LJ14 by the mutant protein was not due to its inadequate production.

In *vitro* activity of ΔC6MtuRRF with homologous EFG (MtuEFG) and heterologous EFG (EcoEFG)

To rule out the possibility that the failure of complementation of *E. coli* LJ14 by ΔC6MtuRRF was due to the temperature-sensitive nature of the mutant per se, we carried out *in vitro* polysome disassembly assays. Fig. 6 shows that the polysome preparation afforded a distinct profile consisting of the monosome and multimer (2×, 3×, 4×, 5×, etc.) peaks on the sucrose gradients (Fig. 6, panel i). When the polysomes were treated with either MtuRRF or MtuEFG no discernible changes in the profiles were observed (Fig. 6a, compare iv and v to i). As expected, when the polysomes were treated with the two proteins together (Fig. 6a, ii and iii), commensurate with their amounts, the polysomes were converted into the monomers. However, ΔC6MtuRRF neither alone (Fig. 6b, iv) nor with EcoEFG (Fig. 6c, ii–iv) resulted in any detectable polysome breakdown. More importantly, and consistent with the *in vivo* analysis, ΔC6MtuRRF

![Image]

Fig. 6. RRF activity assays using *E. coli* polysomes. (a) Assays with MtuRRF and MtuEFG. (i) Polysomes alone, (ii) MtuRRF (10 µg) and MtuEFG (20 µg), (iii) MtuRRF (20 µg) and MtuEFG (40 µg), (iv) MtuRRF (20 µg), and (v) MtuEFG (40 µg). (b) Assays with ΔC6MtuRRF and MtuEFG. (i) Polysomes alone, (ii) ΔC6MtuRRF (20 µg) and MtuEFG (40 µg), (iii) ΔC6MtuRRF (40 µg) and MtuEFG (80 µg), (iv) ΔC6MtuRRF (40 µg), and (v) MtuEFG (80 µg). (c) Assays with mycobacterial and *E. coli* proteins. (i) Polysomes alone, (ii) ΔC6MtuRRF (1 µg) and EcoEFG (4 µg), (iii) ΔC6MtuRRF (4 µg) and EcoEFG (16 µg), (iv) ΔC6MtuRRF (8 µg) and EcoEFG (32 µg), and (v) EcoEFG (2 µg) and EcoEFG (15 µg).
failed to convert the polysomes to monomers even in the presence of homologous EFG (MtuEFG) in spite of the presence of high concentrations of the proteins (Fig. 6b; ii, 20 µg of ∆C6MtuRRF with 40 µg of MtuEFG; iii, 40 µg of ∆C6MtuRRF with 80 µg of MtuEFG). In yet another control experiment, we carried out polysome disassembly assays using EcoRRF to ensure that EcoEFG was active. As seen in Fig. 6(c), EcoRRF converted the polysomes into monosomes in the presence of EcoEFG (Fig. 6c, v). These in vitro assays support the in vivo analysis shown in Fig. 5.

**DISCUSSION**

Termination of protein synthesis releases the nascent polypeptide chains from the ribosomes (Hershey, 1987). However, the mechanistic events that lead to the disassembly of the post-termination complexes and recycling of ribosomes for a fresh round of protein synthesis are still being unravelled. Previous biochemical and genetic analyses have established a key role for RRF in disassembly of the termination complex (Janosi et al., 1996, 2000; Karimi et al., 1999). RRF is unique to prokaryotic protein synthesis, and as demonstrated using *E. coli*, it is essential for the survival of bacteria (Janosi et al., 1994; Inokuchi et al., 2000). However, the gaps in our understanding of the mechanistic aspects of RRF function present a major limitation in the exploitation of this factor as a novel drug target. Mutational analyses, a crucial facet of structure–function studies, are important for a better understanding of the mechanism of action of RRF (Janosi et al., 2000; Fujiwara et al., 2001).

It has been shown that RRF from *P. aeruginosa* complements *E. coli* LJ14 (frr<sup>ts</sup>) (Ohnishi et al., 1999); however, *Tth*RRF does not complement this mutant strain. Interestingly, a mutant lacking five amino acids from the C-terminal end of *Tth*RRF did complement the frr<sup>ts</sup> phenotype of *E. coli* (Fujiwara et al., 1999; Toyoda et al., 2000). Furthermore, recent studies using EcoRRF have shown that deletion of up to seven amino acids from the C-terminal end of this protein still allows it to retain its activity (Fujiwara et al., 2001). Therefore, these C-terminal residues are not absolutely critical for RRF function. Alignment of the available RRF sequences (a total of 49) showed that amino acid residues 178–185 (*E. coli* numbering) belong to a highly conserved stretch of amino acids in RRFs. Does this high degree of conservation allude to the importance of these residues in RRF function?

In this study, based on the sequence comparison of *Mtu*RRF and *Tth*RRF (Fig. 1), we generated a mutant of *Mtu*RRF that lacked the last six amino acids from the C-terminal end (∆C6MtuRRF). However, unlike the C-terminally deleted *Tth*RRF or *Eco*RRF mutants, ∆C6MtuRRF failed to function with *Eco*EFG in ribosome recycling in both in vivo and in vitro analyses. Surprisingly, ∆C6MtuRRF even failed to function with *Mtu*EFG (Figs 5 and 6). The biochemical and biophysical characterizations performed here suggest that the mutant protein is folded properly and retains a shape similar to that of the wild-type protein. Interestingly, the ribosome binding assays (Fig. 4) show that while *Mtu*RRF binds to *E. coli* ribosomes, the mutant protein is compromised for its binding to ribosomes. And, while such binding studies have not been carried out with the equivalent mutants of *Tth*RRF and *Eco*RRF, we suggest that the loss of ribosome binding activity of the ∆C9 mutants of *Eco*RRF (Fujiwara et al., 2001; Toyoda et al., 2000) is predominantly a consequence of the loss of these highly conserved residues at the C-terminal end of the RRF. Thus, the conserved residues at the C-terminal end of the RRFs may facilitate in their direct binding to ribosomes and/or in their prolonged residency on the ribosomes, possibly by modulating the ‘on’ and/or ‘off’ rates.

*T. thermophilus* and *E. coli* belong to the Gram-negative group of bacteria, whereas *M. tuberculosis* belongs to the Gram-positive group, indicating that *Tth*RRF is relatively closer to *Eco*RRF and, therefore, may already possess many of the elements that are needed to establish specific contacts with EFG and the ribosome. Alignment of the RRF sequences (Fig. 1) shows that out of 185 residues a total of 74 are conserved between *Mtu*RRF and *Eco*RRF (∼40% sequence identity) with a similarity score of ∼59% (109/185). However, a total of 81 residues are conserved between *Tth*RRF and *Eco*RRF (∼44% sequence identity) with a similarity score of ∼63% (116/185). Thus, there is slightly higher sequence similarity between *Eco*RRF and *Tth*RRF than between *Eco*RRF and *Mtu*RRF. It has been suggested that a deletion of five amino acids from the C-terminal end of *Tth*RRF results in improved flexibility of the hinge region connecting domains I and II of RRF, which in turn may facilitate its function in *E. coli* (Toyoda et al., 2000). Possibly, what the removal of the residues from the C-terminal end achieves is that it brings these interacting partners into a better configuration for a productive association, such that even the shorter residency times of *Tth*RRF are now adequate. However, in *Mtu*RRF, which in comparison to *Tth*RRF is slightly less intimately related to *Eco*RRF, an equivalent deletion may not result in similar favourable changes. It should also be noted that the earlier study (Fujiwara et al., 1999) utilized a temperature-sensitive strain of *E. coli* containing a different allele of *frr* than the one used in this study. Thus, at this stage alternative interpretations that relate to the strain effects can not be ruled out. Nevertheless, our studies do highlight the significance of RRF binding to ribosomes and the establishment of specific interactions between RRF and EFG for the disassembly of post-termination complexes.

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