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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Ribosome recycling factor (RRF), coded for by the *frr* locus, is involved in the disassembly of post-termination complexes and recycling of the ribosomes for a fresh round of initiation in bacteria and in eukaryotic organelles. In a cross-species-complementation experiment, it was shown that the *Thermus thermophilus* RRF protein lacking five amino acids from its C-terminal end ($\Delta C57thRRF$) but not the full-length protein (*TthRRF*) complemented *Escherichia coli* for its *frr^{ts}* phenotype. It was also shown that the *Mycobacterium tuberculosis* RRF protein (*MtuRRF*) did not complement *E. coli* LJ14 for *frr^{ts}*. However, simultaneous expression of elongation factor G (EFG) and RRF from *M. tuberculosis* resulted in complementation of *E. coli* LJ14. Here it is shown that unlike $\Delta C57thRRF$, an equivalent mutant of *MtuRRF* lacking six amino acids from its C-terminal end ($\Delta C6MtuRRF$) did not complement *E. coli* LJ14. Surprisingly, $\Delta C6MtuRRF$ failed to complement the strain even in the presence of homologous EFG (*MtuEFG*). The biochemical and biophysical characterization of these proteins suggested that the mutant RRF folded properly. However, ribosome-binding assays showed that the mutant protein was compromised in its binding to *E. coli* ribosomes. It is suggested that the conserved amino acids at the C-terminal end of the RRFs contribute to their residency on ribosomes and that the specific interactions between RRF and EFG are crucial in the disassembly of the termination complex.

Keywords: tubercle bacilli, *Thermus thermophilus*, elongation factor G, polysome binding, termination complex

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

Abbreviations: CD, circular dichroism; *EcoEFG*, *E. coli* EFG; *EcoRRF*, full-length *E. coli* RRF; EFG, elongation factor G; *frr*, locus coding for RRF; *frr^{ts}*, locus coding for temperature-sensitive RRF; ESI-MS, electron spray ionization-mass spectroscopy; *MtuEFG*, *M. tuberculosis* EFG; *MtuRRF*, full-length *M. tuberculosis* RRF; RRF, ribosome recycling factor; *TthRRF*, full-length *T. thermophilus* RRF.

Fig. 1. Sequence alignment of MtuRRF, EcoRRF and TthRRF. Conserved residues are highlighted white on a black background; similar residues are shown white on a grey background. Various structural elements in domains I and II (as in EcoRRF) are depicted. The α -helices and β -strands are shown as solid boxes and arrows, respectively. In EcoRRF, domain I consists of residues 1–30 and 104–185, whereas the intervening residues (32–102) constitute domain II (Kim *et al.*, 2000). The sequence alignment was done using PILEUP and CLUSTAL W. BOXSHADE was used to obtain the colour-shaded schematic representation.

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 *frr*^{ts} 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 Δ C5 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 μ g ml⁻¹) and ampicillin (100 μ g 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 0.06% (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 Δ C6MtuRRF. The mutant lacking six amino acids from the C-terminal end of MtuRRF (Δ C6MtuRRF) 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'-GCGCCCATGGTTGATGAGGCTCTCTTC-3') containing an *Nco*I site and a reverse primer (5'-AGCAAAGCTT-ATTCTTTGTGTTTAACC-3') which incorporated a stop codon at position 180 (G to amber) and a *Hind*III site. The

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

Strain/plasmid	Relevant details*	Reference
Strain		
LJ14	MC1061 containing <i>frr14^{ts}</i> allele	Janosi <i>et al.</i> (1998)
MRE600	F ⁻ , <i>rna</i>	Gay (1984)
BL21(DE3)	<i>hsdS gal</i> (Δ <i>clts857 ind1 sam7 nin5 lacUV5-T7</i> gene 1)	Studier & Moffatt (1986)
Plasmid		
pACDH	Harbours ACYC origin of replication; derived from pACD (Mangroo <i>et al.</i> , 1995) by mutating the <i>Hind</i> III site of the Tet ^R marker to the <i>Nhe</i> I site	T. K. DineshKumar & U. Varshney (unpublished data)
pACDH <i>Mtu</i> RRF	<i>M. tuberculosis</i> RRF cloned into pACDH	Rao & Varshney (2001)
pACDH Δ C6 <i>Mtu</i> RRF	<i>M. tuberculosis</i> RRF (Δ C6 mutant) cloned into pACDH	This work
pTrc <i>Eco</i> EFG	<i>E. coli</i> EFG cloned into pTrc99C	Rao & Varshney (2001)
pTrc <i>Mtu</i> EFG	<i>M. tuberculosis</i> EFG cloned into pTrc99C	Rao & Varshney (2001)
pET <i>Mtu</i> RRF	pET11d-based overexpression construct of <i>Mtu</i> EFG	Rao & Varshney (2001)
pET Δ C6 <i>Mtu</i> RRF	pET11d-based overexpression construct of Δ C6 <i>Mtu</i> RRF	This work
pET <i>Eco</i> RRF	pET11d-based overexpression construct of <i>Eco</i> RRF	Rao & Varshney (2001)
pET <i>Eco</i> EFG	pET11d-based overexpression construct of <i>Eco</i> EFG	Rao & Varshney (2001)
pET <i>Mtu</i> EFG	pET11d-based overexpression construct of <i>Mtu</i> EFG	Rao & Varshney (2001)

* Tet^R, tetracycline-resistant.

PCR product was digested with *Nco*I and *Hind*III and cloned into the respective sites of the ColE1 origin-of-replication-based vectors pTrc99C and pET11d to generate pTrc Δ C6*Mtu*RRF and pET Δ C6*Mtu*RRF, respectively. The same fragment was also subcloned into pACDH containing a pACYC origin of replication to yield pACDH Δ C6*Mtu*RRF. Incorporation of the fragment was confirmed by complete DNA sequencing.

Purification of *Mtu*RRF and Δ C6*Mtu*RRF, N-terminal sequencing and electron spray ionization-mass spectroscopy (ESI-MS). *Mtu*RRF and Δ C6*Mtu*RRF were purified from *E. coli* BL21(DE3) harbouring either pET*Mtu*RRF or pET Δ C6*Mtu*RRF, respectively. The transformants were inoculated into 2 $\times$ YT (Sambrook *et al.*, 1989) (2 l) and induced with 0.5 mM IPTG at the mid-exponential phase of growth (OD₆₀₀ value of between 0.3 and 0.4). Cells were harvested, sonicated and used to make 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, dialysed 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* EFG. EFGs were purified from *E. coli* BL21(DE3) using the T7 RNA polymerase

expression constructs pET*Mtu*EFG and pET*Eco*EFG (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 (OD₂₆₀ ~2) were incubated in reaction volumes of 250 μ l without or with RRFs in the RRF assay buffer (10 mM Tris/HCl, pH 7.4, 80 mM NH₄Cl, 8.2 mM MgSO₄, 1 mM DTT, 10 μ M puromycin, 160 μ M 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 μ g 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-*Mtu*RRF rabbit antibodies and detected using alkaline phosphatase-conjugated goat-anti-rabbit IgG with the substrates *p*-nitrotertrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate.

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,

Fig. 2. Purification and characterization of RRFs. (a) Purification profile of *Mtu*RRF on SDS-PAGE (15%). Lanes: 1 and 2, aliquots (~8 μ g) from S20 and S100 extracts, respectively; 3–5, aliquot (~4 μ g) from the pool of eluates from Superdex 75, Mono Q and hydroxyapatite columns, respectively. (b) Analysis of purified RRFs (~4 μ g each protein) on SDS-PAGE (15%). Lanes: 1, molecular mass marker (Sigma); 2, Δ C6*Mtu*RRF; 3, *Eco*RRF; 4, *Mtu*RRF. (c) Analysis of purified RRFs on 15% PAGE (native). Lanes: 1, Δ C6*Mtu*RRF (~2 μ g); 2, *Eco*RRF (~2 μ g); 3, *Mtu*RRF (~3 μ g).

Fig. 3. CD spectroscopy. (a) Secondary structure and (b) tertiary structure CD spectra of *Mtu*RRF (●, a; ○, b) and Δ C6*Mtu*RRF (○, a; ●, b).

Fig. 4. Binding of *Mtu*RRF and Δ C6*Mtu*RRF to *E. coli* polysomes. The binding to polysomes was analysed using a spin-column method (see Methods). Lanes: 1, Δ C6*Mtu*RRF alone (5 μ g); 2, Δ C6*Mtu*RRF alone (50 μ g); 3, *Mtu*RRF (5 μ g) and Δ C6*Mtu*RRF (5 μ g); 4, *Mtu*RRF (5 μ g) and Δ C6*Mtu*RRF (25 μ g); 5, *Mtu*RRF (5 μ g) and Δ C6*Mtu*RRF (50 μ g); 6, *Mtu*RRF (5 μ g); 7, polysomes alone; 8, *Mtu*RRF (5 μ g) and Δ C6*Mtu*RRF (50 μ g) without polysomes; M1 and M2, marker lanes for *Mtu*RRF and Δ C6*Mtu*RRF, respectively.

?DEALFDAEE..., which matched with the N-terminal of *Mtu*RRF sequence deduced from its gene, suggesting that the initiator f^{Met} 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 f^{Met}. However, in this analysis, a minor peak of 20851 Da corresponding to a polypeptide in which f^{Met} had not been removed was also seen. This was most likely due to hyperexpression of *Mtu*RRF.

Fig. 5. Analysis of complementation of *E. coli* LJ14 (*frr*^{ts}) with Δ C6MtuRRF. (a) Various plasmids were introduced into *E. coli* LJ14 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, pACDHΔC6MtuRRF and pTrcMtuEFG. (b) Growth of *E. coli* LJ14 (*frr*^{ts}) 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 Δ C6MtuRRF expression in *E. coli* LJ14 by immunoblotting using anti-MtuRRF antibodies. Protein extracts (10 μ g total protein) prepared from cultures

Δ C6MtuRRF 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, Δ C6MtuRRF 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 Δ C6MtuRRF, 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; Toyada *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 (Δ C6MtuRRF) 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 Δ C6MtuRRF and MtuRRF bound to ribosomes (Fig. 4, lanes 1 and 6, respectively), the binding of Δ C6MtuRRF was weaker than that of MtuRRF. However, when Δ C6MtuRRF was taken in a large amount (50 μ g), its binding to ribosomes was comparable to that of the full-length protein (5 μ g) (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).

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.

Fig. 6. RRF activity assays using *E. coli* polysomes. (a) Assays with *MtuRRF* and *MtuEFG*. (i) Polysomes alone, (ii) *MtuRRF* (10 μ g) and *MtuEFG* (20 μ g), (iii) *MtuRRF* (20 μ g) and *MtuEFG* (40 μ g), (iv) *MtuRRF* (20 μ g), and (v) *MtuEFG* (40 μ g). (b) Assays with Δ C6*MtuRRF* and *MtuEFG*. (i) Polysomes alone, (ii) Δ C6*MtuRRF* (20 μ g) and *MtuEFG* (40 μ g), (iii) Δ C6*MtuRRF* (40 μ g) and *MtuEFG* (80 μ g), (iv) Δ C6*MtuRRF* (40 μ g), and (v) *MtuEFG* (80 μ g). (c) Assays with mycobacterial and *E. coli* proteins. (i) Polysomes alone, (ii) Δ C6*MtuRRF* (1 μ g) and *EcoEFG* (4 μ g), (iii) Δ C6*MtuRRF* (4 μ g) and *EcoEFG* (16 μ g), (iv) Δ C6*MtuRRF* (8 μ g) and *EcoEFG* (32 μ g), and (v) *EcoRRF* (2 μ g) and *EcoEFG* (15 μ g).

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 μ g) and increasing amounts of Δ C6*MtuRRF* (5 to 50 μ g), 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 Δ C6*MtuRRF* to bind to the ribosomes further supported the view that it folded correctly.

Complementation analysis of *E. coli* LJ14 with Δ C6*MtuRRF*

As the deletion of the C-terminal five amino acids from *TthRRF* bestowed upon it the ability to complement an *frr^{ts}* strain of *E. coli*, it was of interest to us to investigate the phenotype of an equivalent mutant of *MtuRRF*. However, our analysis showed that Δ C6*MtuRRF* failed to rescue the temperature-sensitive phenotype of *E. coli* LJ14 (*frr^{ts}*) 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 Δ C6*MtuRRF* to rescue the temperature-sensitive phenotype of *E. coli* LJ14 at the non-permissive temperature (Fig. 5a, sector 3). In fact, Δ C6*MtuRRF* 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 *MtuRRF* 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 Δ C6*MtuRRF* 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 Δ C6*MtuRRF* 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 Δ C6*MtuRRF* produced upon induction with IPTG (Fig. 5c, lanes 2, 4 and 6) were comparable to those of wild-type *MtuRRF* 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 Δ C6*MtuRRF* with homologous EFG (*MtuEFG*) and heterologous EFG (*EcoEFG*)

To rule out the possibility that the failure of complementation of *E. coli* LJ14 by Δ C6*MtuRRF* 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 $\times$, 3 $\times$, 4 $\times$, 5 $\times$, 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, Δ C6*MtuRRF* 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, Δ C6*MtuRRF*

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 $\Delta C6MtuRRF$ with 40 µg of *MtuEFG*; iii, 40 µg of $\Delta C6MtuRRF$ 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^{ts}*) (Ohnishi *et al.*, 1999); however, *TthRRF* does not complement this mutant strain. Interestingly, a mutant lacking five amino acids from the C-terminal end of *TthRRF* did complement the *frr^{ts}* 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 *MtuRRF* and *TthRRF* (Fig. 1), we generated a mutant of *MtuRRF* that lacked the last six amino acids from the C-terminal end ($\Delta C6MtuRRF$). However, unlike the C-terminally deleted *TthRRF* or *EcoRRF* mutants, $\Delta C6MtuRRF$ failed to function with *EcoEFG* in ribosome recycling in both *in vivo* and *in vitro* analyses. Surprisingly, $\Delta C6MtuRRF$ even failed to function with *MtuEFG* (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 *MtuRRF* 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 *TthRRF* and *EcoRRF*, we suggest that the loss of ribosome binding activity of the $\Delta C9$ mutants of *EcoRRF* (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 *TthRRF* is relatively closer to *EcoRRF* 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 *MtuRRF* and *EcoRRF* (~40% sequence identity) with a similarity score of ~59% (109/185). However, a total of 81 residues are conserved between *TthRRF* and *EcoRRF* (~44% sequence identity) with a similarity score of ~63% (116/185). Thus, there is slightly higher sequence similarity between *EcoRRF* and *TthRRF* than between *EcoRRF* and *MtuRRF*. It has been suggested that a deletion of five amino acids from the C-terminal end of *TthRRF* 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 *TthRRF* are now adequate. However, in *MtuRRF*, which in comparison to *TthRRF* is slightly less intimately related to *EcoRRF*, 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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