Specific interaction between the ribosome recycling factor and the elongation factor G from *Mycobacterium tuberculosis* mediates peptidyl-tRNA release and ribosome recycling in *Escherichia coli*

Arasada Rajeswara Rao and
Umesh Varshney¹

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, 560 012, India

¹Corresponding author
e-mail: varshney@mcbl.iisc.ernet.in

Once the translating ribosomes reach a termination codon, the nascent polypeptide chain is released in a factor-dependent manner. However, the P-site-bound deacylated tRNA and the ribosomes themselves remain bound to the mRNA (post-termination complex). The ribosome recycling factor (RRF) plays a vital role in dissociating this complex. Here we show that the *Mycobacterium tuberculosis* RRF (*Mtu*RRF) fails to rescue *Escherichia coli* LJ14, a strain temperature-sensitive for RRF (*frr*^{ts}). More interestingly, co-expression of *M.tuberculosis* elongation factor G (*Mtu*EFG) with *Mtu*RRF rescues the *frr*^{ts} strain of *E.coli*. The simultaneous expression of *Mtu*EFG is also needed to cause an enhanced release of peptidyl-tRNAs in *E.coli* by *Mtu*RRF. These observations provide the first genetic evidence for a functional interaction between RRF and EFG. Both the *in vivo* and *in vitro* analyses suggest that RRF does not distinguish between the translating and terminating ribosomes for their dissociation from mRNA. In addition, complementation of *E.coli* PEM100 (*fusA*^{ts}) with *Mtu*EFG suggests that the mechanism of RRF function is independent of the translocation activity of EFG.

Keywords: *frr/fusA*/mycobacteria/peptidyl-tRNA/termination of protein synthesis

Introduction

In eubacteria, once the translating ribosomes reach a termination codon, binding of RF1 in response to UAA and UAG, or RF2 in response to UAA and UGA into the ribosomal A-site triggers hydrolysis of the P-site-bound peptidyl-tRNA culminating in the release of the nascent polypeptide chain (Hershey, 1983). Several studies have now shown that RF3 binding to the ribosomal A-site catalyses recycling of RF1 and RF2 (Buckingham *et al.*, 1997; Pavlov *et al.*, 1997b). However, subsequent to the release of polypeptide, the 70S ribosome and the P-site-bound deacylated tRNA still remain attached to the mRNA. The most important events in initiation of protein synthesis occur on the P-site of the 30S ribosomal subunits (Hershey, 1983; Kozak, 1983). Therefore, it is imperative that the terminating ribosomal complex dissociates before the start of a fresh round of polypeptide biosynthesis. Mechanistic details of this post-termination step (also

termed as the 'fourth step' of protein synthesis) have remained largely unknown. The pioneering work of Kaji and others suggested the involvement of a new factor, ribosome recycling factor (RRF), in dissociation of the termination complex (Hirashima and Kaji, 1972; Subramanian and Davis, 1973). Using *in vitro* assays, it was also demonstrated that a highly purified preparation of RRF was inadequate to dissociate the model substrates. However, when the RRF preparation contained elongation factor G (EFG), it converted the polysomes into monosomes in the presence of GTP (Hirashima and Kaji, 1972). Subsequent work demonstrated that RRF is vital for cellular metabolism and, in *Escherichia coli*, it has already been shown to be an essential protein (Janosi *et al.*, 1994). RRF appears to be unique to the prokaryotic world.

This observation has stimulated a great deal of research on RRF in recent times, and crystal structures of RRF from *E.coli*, *Thermotoga maritima* and *Thermus thermophilus* have been determined recently (Selmer *et al.*, 1999; Kim *et al.*, 2000; Toyoda *et al.*, 2000). Interestingly, the crystal structures reveal that RRF is a remarkable mimic of tRNA. This observation as well as an *in vitro* study suggested that similarly to its action at the elongation step, EFG translocates RRF from the ribosomal A-site to the P-site, which in turn dissociates the post-termination complexes (Pavlov *et al.*, 1997a; Selmer *et al.*, 1999). On the contrary, a recent elegant biochemical study which utilized short synthetic mRNAs suggested that the EFG function at the post-termination step is distinct from its classical role at the translocation step (Karimi *et al.*, 1999). Both EFG and RRF are essential proteins and, therefore, demonstration of the relevance of these *in vitro* studies and various other theoretical models (Janosi *et al.*, 1996) of RRF function in the cellular context has remained a challenging task.

We are interested in exploring the mechanism of protein synthesis in *E.coli* and in *Mycobacterium tuberculosis*, a Gram-positive (acid-fast) eubacterium that afflicts nearly one-third of the world's population and is a major cause of human death worldwide. In this study, by simultaneous expression of RRF and EFG from *M.tuberculosis* in *E.coli*, we provide the first genetic evidence for the functional interaction between RRF and EFG *in vivo*. More importantly, this assay system has allowed us to explore the mechanism of RRF action in the cellular milieu and discriminate among the various models proposed for RRF action (Janosi *et al.*, 1996).

Results

*Mtu*RRF alone does not rescue the temperature-sensitive phenotype of *E.coli* LJ14 (*frr*^{ts})

The comparison of the primary sequences of *Eco*RRF and *Mtu*RRF (Figure 1A) shows an ~40% identity and 55% similarity between the two proteins. However, when we

Fig. 1. (A) Comparison of the primary structures of *E.coli* RRF (EcoRRF) and *M.tuberculosis* RRF (MtuRRF). Shadings in black and grey indicate identical and similar residues, respectively. (B) Analysis of complementation of *E.coli* LJ14 (*frr^{ts}*) with MtuRRF. Various plasmids were introduced into *E.coli* LJ14 and the transformants were streaked on LB agar plates containing ampicillin and IPTG in duplicate. One plate was incubated at the permissive temperature (30°C) and the other at the non-permissive temperature (42°C). Sectors: 1 and 2, pTrc99C vector; 3 and 4, pTrcMtuRRF; 5 and 6, pTrcEcoRRF. (C) Detection of MtuRRF expression in *E.coli* LJ14 by immunoblotting using anti-MtuRRF antibodies. Protein extracts were prepared from transformants harbouring either the pTrcMtuRRF expression construct (lanes 1 and 2) or the pTrc99C vector (lanes 3 and 4). Cultures were grown in the presence (+) or absence (–) of IPTG. (D) Growth curves of various transformants of *E.coli* LJ14. Fresh cultures were started by inoculating with overnight cultures (0.06%) from 30°C, and grown at the permissive (30°C, open symbols) and non-permissive (42°C, filled symbols) temperatures. The OD at 600 nm (y-axis) was monitored at regular intervals (x-axis). The presence of pTrc99C or its recombinants is indicated in the inset.

carried out the complementation analysis in the *E.coli* LJ14 (*frr^{ts}*) strain using the *MtuRRF* gene cloned onto a medium copy expression vector (pTrc*MtuRRF*), it failed to complement the RRF^{ts} phenotype of the strain (Figure 1B, 42°C, sectors 3 and 4). As expected, under the same conditions, expression of *EcoRRF* (pTrc*EcoRRF*) complemented the strain (sectors 5 and 6). Immunoblot analysis of the cell-free extracts of the transformants grown at the permissive temperature (30°C) showed that *MtuRRF* was produced in the LJ14 strain (Figure 1C, lanes 1 and 2). This observation ruled out the lack of *MtuRRF* expression as a possible reason for the failure to restore the growth of the strain at the non-permissive temperature (42°C). To investigate further, we determined the growth rates of the various transformants in liquid culture at the permissive and non-permissive temperatures (Figure 1D). At the permissive temperature, transformants harbouring pTrc*EcoRRF* or pTrc*MtuRRF* grew at the same rate as those harbouring the vector alone, suggesting that the expression of these RRFs was not toxic to *E.coli*. However, at the non-permissive temperature (42°C), while the transformants harbouring pTrc*EcoRRF* grew well, those harbouring either the vector or pTrc*MtuRRF* did not. This observation confirms the results shown in Figure 1B, and suggests that *MtuRRF* alone does not complement the LJ14 strain to any detectable level.

***MtuRRF* requires *MtuEFG* to rescue the temperature-sensitive phenotype of *E.coli* LJ14 (*frr^{ts}*)**

In vitro experiments performed with RRF have revealed that for its function it requires EFG (Janosi *et al.*, 1996). Therefore, we reasoned that the presence of *MtuEFG* might facilitate complementation of the LJ14 strain with *MtuRRF*. Interestingly, when *MtuRRF* and *MtuEFG* were expressed simultaneously from the two compatible plasmids, pACDH*MtuRRF* and pTrc*MtuEFG*, in *E.coli* LJ14, the temperature-sensitive phenotype of the strain was rescued and it grew well at the non-permissive temperature (42°C) (Figure 2A, sectors 7 and 8). However, neither *MtuRRF* (sectors 3 and 4) nor *MtuEFG* (sectors 5 and 6) alone complemented the strain. Further, as shown in Figure 2B, overproduction of *EcoEFG* with *MtuRRF* (compare sectors 5 and 6, with sectors 3 and 4 at 42°C) also did not rescue the temperature-sensitive phenotype of the LJ14 strain. The growth curves (Figure 2C) showed that simultaneous expression of *MtuRRF* and *MtuEFG* completely alleviated the temperature-sensitive phenotype of *E.coli* LJ14. Thus, these observations provide genetic evidence for a functional interaction between RRF and EFG.

MtuEFG* participates at the translocation step in *E.coli

To investigate the mechanism of functional interaction between EFG and RRF, we introduced the *MtuEFG* expression vector into *E.coli* PEM100. This strain possesses a mutation in the *fusA* gene, which renders it temperature sensitive because the encoded EFG (G502D) is defective at the translocation step (Hou *et al.*, 1994). All the transformants (pTrc99C, pTrc*EcoEFG* and pTrc*MtuEFG*) grew at the permissive temperature (Figure 3A, 30°C). Interestingly, both *EcoEFG* and

MtuEFG rescued the temperature-sensitive phenotype of the strain (Figure 3A, sectors 2 and 3, 42°C).

In yet another experiment, we overproduced *EcoEFG* or *MtuEFG* in *E.coli* (wild-type for *fusA*) and checked if they resulted in a similar response to fusidic acid. Fusidic acid is a classical inhibitor, which affects the translocation function of EFG by freezing it onto ribosomes in its GDP-bound form (Rodnina and Wintermeyer 1998). Interestingly, when compared with the vector control, the overproduction of *EcoEFG* in cells made them hypersensitive to fusidic acid (Figure 3B, compare sectors 5 and 6 with sectors 1 and 2 in the panels on the left with no fusidic acid with the panel in the centre with 25 µg/ml fusidic acid or the panel on the right with 50 µg/ml fusidic acid). Further, similarly to *EcoEFG*, expression of *MtuEFG* also resulted in hypersensitivity of the host to fusidic acid (sectors 3 and 4). While the exact mechanism of hypersensitivity of *E.coli* to fusidic acid upon overproduction of EFGs is not clear, it could be that the high occupancy of ribosomes with the EFG-GDP complex (Agrawal *et al.*, 1998) in the presence of the inhibitor causes toxicity to cells. Nevertheless, the observation that overproduction of either of the EFGs makes *E.coli* hypersensitive to fusidic acid further supports that the mycobacterial protein can substitute for the function of *EcoEFG*.

***In vitro* conversion of polysomes to monosomes by RRF and EFG**

To further our understanding of the role of *MtuRRF* and *MtuEFG* in ribosome recycling, we used an established method described by Kaji and co-workers (Ohnishi *et al.*, 1999) for the *in vitro* activity assay of RRF, wherein conversion of polysomes to monosomes was monitored (Figure 4A and B). Factor-free polysomes were prepared from *E.coli* MRE600 and, under the experimental conditions, the profile of the buffer control shows a clear separation between the monosomes and the higher forms of the polysomes in the preparation (panels marked a). The controls wherein the polysomes were treated with RRF or EFG alone also show a similar profile (panels c and d). When the polysomes were treated with *EcoRRF* and *EcoEFG*, the polysomes were converted into monosomes (Figure 4A, b). Similarly, as expected from the *in vivo* complementation data, treatment of *E.coli* polysomes with the *M.tuberculosis* proteins (RRF and EFG) also resulted in enrichment of the monosome peak (Figure 4B, b). Interestingly, neither of the heterologous combinations of proteins (*EcoRRF* with *MtuEFG* or *MtuRRF* with *EcoEFG*) showed any activity in the conversion of polysomes to monosomes (Figure 4B, e). The findings of this experiment corroborate those of the *in vivo* experiments (Figures 1 and 2) and, taken together, clearly demonstrate that RRF, for its function, requires EFG from homologous sources.

Role of RRF and EFG in peptidyl-tRNA release from the translating ribosomes in vivo

Not all the peptide chains that begin from the initiation codon of the mRNA give rise to a full-length protein. Even under normal physiological conditions, peptidyl-tRNAs are released from the ribosomes during chain elongation. As the accumulation of peptidyl-tRNAs is toxic, cells

Fig. 2. (A) Analysis of complementation of *E. coli* LJ14 (*frr*^{ts}) with *MtuRRF*. Various plasmids were introduced into *E. coli* LJ14 and the transformants were streaked on LB agar plates containing ampicillin, tetracycline and IPTG in duplicate. One plate was incubated at the permissive temperature (30°C) and the other at the non-permissive temperature (42°C). Sectors: 1 and 2, pTrc99C and pACDH vectors; 3 and 4, pTrc99C and pACDH*MtuRRF*; 5 and 6, pTrc*MtuEFG* and pACDH; 7 and 8, pTrc*MtuEFG* and pACDH*MtuRRF*. (B) Analysis of complementation of *E. coli* LJ14 (*frr*^{ts}) with *MtuRRF* along with *EcoEFG*. The transformants were streaked on LB agar plates containing ampicillin, tetracycline and IPTG in duplicate. One plate was incubated at the permissive temperature (30°C) and the other at the non-permissive temperature (42°C). Sectors: 1 and 2, pTrc99C and pACDH vector control; 3 and 4, pTrc*MtuEFG* and pACDH*MtuRRF*; 5 and 6, pTrc*EcoEFG* and pACDH*MtuRRF*. (C) Growth of *E. coli* LJ14 (*frr*^{ts}) harbouring various plasmids at the permissive (30°C) and non-permissive (42°C) temperatures. *Escherichia coli* LJ14 harbouring various plasmid constructs were grown overnight at the permissive temperature (30°C). Fresh cultures in LB containing ampicillin, tetracycline and IPTG were started by inoculating (0.06%) with overnight cultures from 30°C, and grown at the permissive (30°C, open symbols) and non-permissive (42°C, filled symbols) temperatures. The OD at 600 nm (y-axis) was monitored at regular intervals (x-axis). The inset shows the proteins that were overproduced from the pACDH or pTrc99C vectors. pTrc*MtuEFG* and pACDH (open and closed circles), pTrc*MtuEFG* and pACDH*MtuRRF* (open and closed inverted triangles).

contain peptidyl-tRNA hydrolase (PTH), which hydrolyses the peptidyl-tRNA ester bond (Kossel and RajBhandary, 1968). Conditional mutants of *E. coli* (*pth*^{ts}) have been isolated, which grow at 30°C (permissive temperature) but not at 37°C (non-permissive temperature) (Atherly and Menninger, 1972; Menninger *et al.*, 1973). Recently, in a genetic screen, it was found that the suppressors of *pth*^{ts} mapped to the promoter region of *frr*, resulting in low levels of RRF in the cell (Heurgue-Hamard *et al.*, 1998). This suggests that RRF can

somehow cause premature release of peptidyl-tRNAs from the ribosome.

In the experiment shown in Figure 5A, by making use of the acid urea gels (Varshney *et al.*, 1991a), we examined the consequence of overproduction of *EcoRRF* and *MtuRRF* in *E. coli* AA7852 (*pth*^{ts}) on the accumulation of peptidyl-tRNAs. These gels allow direct analysis of the *in vivo* status of the tRNAs by separating the aminoacylated and uncharged forms. However, when peptides of heterogeneous length are attached to the tRNA, the

Fig. 3. (A) Analysis of complementation of *E.coli* PEM100 (*fusA*^{ts}) with *MtuEFG*. The PEM100 strain was transformed with various plasmids and the transformants streaked in duplicate on LB agar containing ampicillin and IPTG. One plate was incubated at the permissive temperature (30°C) and the other at the non-permissive temperature (42°C). Sectors: 1, pTrc99C; 2, pTrc*MtuEFG*; 3, pTrc*EcoEFG*. **(B)** Analysis of growth of *E.coli* TG1 containing various plasmids on LB agar containing ampicillin and IPTG alone (left) or with 25 µg/ml (centre) and 50 µg/ml (right) fusidic acid, respectively. Sectors: 1 and 2, pTrc99C; 3 and 4, pTrc*MtuEFG*; 5 and 6, pTrc*EcoEFG*.

aminoacylated form of the tRNA is converted into a smear. As expected, the transformants grown at 30°C (permissive temperature) do not show accumulation of peptidyl-tRNAs in the cell (lanes 1–5, absence of a smear above the Tyr-tRNA^{Tyr} band). However, at 34°C, the temperature at which PTH is partially inactive, minimal accumulation of peptidyl-tRNA (as detected by the smear corresponding to peptidyl-Tyr-tRNA^{Tyr}) was seen in the vector control upon overexposure of the autoradiogram (Figure 5A, lane 6, lower panel). Overproduction of *EcoRRF* resulted in the accumulation of peptidyl-tRNA at 34°C (lane 10). More importantly, while overproduction of *MtuRRF* alone (lane 8) or *MtuEFG* alone (lane 9) had no effect on accumulation of peptidyl-tRNA over and above the vector-alone background (lane 6), their simultaneous expression resulted in an increased accumulation of peptidyl-tRNA in cells (lane 7). Thus, in the context of *E.coli* ribosomes, *MtuRRF* participated even in enhanced release of peptidyl-tRNA and, at this step also, it required the presence of EFG from the homologous source. Taken together, these results point to a unified mechanism of RRF action both at the post-termination and the peptidyl-tRNA release steps. Furthermore, to examine the effect of the accumulation of peptidyl-tRNA, we determined the

growth rates of the various transformants (Figure 5B). The cells harbouring the *EcoRRF* plasmid, as expected, grew slowly in the liquid medium. Similarly, the cells harbouring the *EcoEFG* plasmid also grew slowly. On the other hand, the transformants harbouring either the *MtuRRF* or the *MtuEFG* plasmids grew just as well as the vector control. More interestingly, the transformants harbouring both the *MtuRRF* and *MtuEFG* plasmids grew slowly, most probably as a consequence of the accumulation of the peptidyl-tRNA (Figure 5A, lane 7).

Discussion

Translation termination and the post-termination steps that lead to recycling of ribosomes remain one of the least understood aspects of protein biosynthesis (Janosi *et al.*, 1996; Kisselev and Buckingham, 2000). However, the three-dimensional structure determination of RRF from *T.maritima*, *T.thermophilus* and *E.coli*, and recent biochemical studies have led to an unprecedented stimulation and advancement of research in these areas (Pavlov *et al.*, 1997a,b; Janosi *et al.*, 1998; Karimi *et al.*, 1999; Selmer *et al.*, 1999; Inokuchi *et al.*, 2000; Kim *et al.*, 2000; Toyoda *et al.*, 2000). Accordingly, the current view of the

Fig. 4. RRF activity assays. Reactions were set up as described in Materials and methods. (A) (a) Polysomes alone; (b) *EcoRRF* and *EcoEFG* (15 µg each); (c) *EcoEFG* (30 µg); (d) *EcoRRF* (15 µg); (e) *MtuRRF* and *EcoEFG* (15 and 30 µg, respectively). (B) (a) Polysomes alone; (b) *MtuRRF* and *MtuEFG* (15 and 30 µg, respectively); (c) *MtuEFG* (30 µg); (d) *MtuRRF* (15 µg); (e) *EcoRRF* and *MtuEFG* (15 and 30 µg, respectively).

post-termination events that lead to dissociation of the termination complex suggests that subsequent to the peptide chain release by RF1/RF2, RF3 mediates the eviction of these release factors in a GTP hydrolysis-dependent process, and the subsequent action of RRF and EFG leads to the dissociation of the post-termination complex. The *in vitro* experiments (Pavlov *et al.*, 1997a) suggested that for ribosome recycling, EFG might be involved in translocation of RRF from the ribosomal A-site to the P-site (similar to the translocation of the peptidyl-tRNA). This hypothesis received a boost from the three-dimensional structure of RRF, which showed it to be a spectacular example of protein mimicry of a tRNA molecule (Selmer *et al.*, 1999). However, recently, an elegant biochemical study suggested that the function of EFG at this step is distinct from that of its involvement at the translocation step (Karimi *et al.*, 1999).

Recently, it was shown that the RRF from *Pseudomonas aeruginosa* complements *E. coli* LJ14 (Ohnishi *et al.*, 1999). On the other hand, the RRF from *T. thermophilus* does not complement the temperature-sensitive phenotype

of *E. coli* (*frr*^{ts}). However, a mutant that lacked five amino acids from the C-terminal end of the *T. thermophilus* RRF did complement the *E. coli* for *frr*^{ts} (Fujiwara *et al.*, 1999; Toyoda *et al.*, 2000). Surprisingly, in our studies with the *M. tuberculosis* RRF, neither the wild-type (Figure 1) nor a mutant protein that lacked the C-terminal five amino acids complemented the *E. coli* *frr*^{ts} strain (data not shown). More importantly, we discovered that simultaneous expression of both the *MtuRRF* and *MtuEFG* (but not *EcoEFG*) rescued the temperature-sensitive phenotype of *E. coli* (*frr*^{ts}). This observation has allowed us to use *E. coli* as a surrogate host to investigate the mechanism of action of RRF *in vivo*. As we failed to detect an interaction between *MtuEFG* and *MtuRRF* by surface plasmon resonance and far western analysis (data not shown), it seems unlikely that EFG escorts RRF to the ribosomes. Most probably, specific interactions between the two proteins are effected on the ribosomes, and the appropriateness of these interactions is necessary for their biological activity. Recently, it was shown that the overproduction of RRFs from heterologous sources resulted in toxicity to *E. coli* (Atarashi and Kaji, 2000). A possible explanation for the observed toxicity could be that these proteins, by virtue of their structural mimicry of tRNA, occupy the ribosomal A-site but fail to establish functional interaction with *EcoEFG*. The formation of such 'dead' complexes could be deleterious for the growth of *E. coli*. It would be interesting to study whether the simultaneous expression of EFGs from homologous sources could alleviate the toxic effects of the heterologous RRFs.

To gain further insights into the mechanism of EFG participation in ribosome recycling, we used *E. coli* PEM100 (*fusA*^{ts}). This strain carries a G502D mutation in the EFG, which makes it defective in translocation at the non-permissive temperature (Hou *et al.*, 1994). Recently, several other mutants (H583K, H583R, etc.) in this region (domain 4) of *EcoEFG* were also demonstrated to be defective in translocation (Savelsbergh *et al.*, 2000). However, the properties of these mutants in ribosome binding, interaction with the α -sarcin loop of the 23S rRNA, GTP hydrolysis and P_i release were unaffected (Savelsbergh *et al.*, 2000). Interestingly, the *MtuEFG* complements *E. coli* PEM100, a *fusA*^{ts} strain, suggesting that the translocation function of the *EcoEFG* and *MtuEFG* is conserved. On the other hand, as shown by the polysome dissociation assay (Figure 4), *MtuEFG* fails to collaborate with *EcoRRF* and, since the ribosome recycling is an essential function, the survival of the PEM100 strain in the presence of *MtuEFG* at the non-permissive temperature raises an intriguing question. How do these cells carry out ribosome recycling? We can offer two possible explanations: (i) the resident *EcoEFG* (chromosomally encoded) must still be able to function at the ribosome recycling step by a translocation-independent mechanism; or (ii) RF3, a classical G-protein that shares homology with EFG but lacks domain 4 (important in translocation) and binds to ribosomes, may substitute for the function of EFG (Grentzmann *et al.*, 1998). Therefore, in either case, the mechanism of ribosome dissociation has to be independent of the translocation-like function of EFG. Thus it is appealing to speculate that the 'conformational tickling' following the hydrolysis of the factor-bound GTP may

Fig. 5. (A) Analysis of the accumulation of peptidyl-tRNA in *E. coli* AA7852 (*pth^{ts}*). The *E. coli pth^{ts}* (AA7852) was transformed with various plasmid constructs, grown in LB containing ampicillin, tetracycline and IPTG at the permissive (30°C) or partially non-permissive (34°C) temperature and used to prepare total tRNA under acidic phenol conditions. Total tRNA was fractionated on an acid urea gel, electroblotted onto a nytran membrane and hybridized with 5'-³²P-end-labelled anti-tRNA^{Tyr} probe. Photographs of autoradiographs with short (upper panel) and longer (lower panel) exposures. -, pACDH vector; M, pACDH*MtuRRF*; and E, pTrc*EcoRRF* in the RRFs row; and -, pTrc99C; and M, pTrc*MtuEFG* in the EFG row, respectively. Temperatures at which the cultures were grown are as indicated. **(B)** Analysis of growth of *E. coli* AA7852 (*pth^{ts}*) harbouring various plasmids in LB medium containing ampicillin, tetracycline and IPTG grown at the partially non-permissive temperature (35°C). pTrc99C and pACDH vectors (closed circles); pTrc99C and pACDH*MtuRRF* (open circles); pTrc*MtuEFG* and pACDH (closed inverted triangles); pTrc*EcoEFG* and pACDH (open squares); pTrc*EcoRRF* and pACDH (open inverted triangles); and pTrc*MtuEFG* and pACDH*MtuRRF* (closed squares).

constitute a critical aspect of RRF-mediated ribosome recycling.

Yet another aspect of RRF activity that we have investigated by using *E. coli* as a surrogate host is its involvement in the step of release of peptidyl-tRNAs from ribosomes. Although the accumulation of peptidyl-tRNAs is toxic to cells, for fidelity in translation the peptidyl-tRNA release from ribosomes constitutes an important aspect of protein biosynthesis. Therefore, the

peptidyl-tRNA hydrolase, important in recycling of the tRNAs, is an essential enzyme. The observation that the suppressors of *pth^{ts}* map to the promoter region of *frr* (down-regulation) was of great significance in establishing a role for RRF at this step (Heurgue-Hamard *et al.*, 1998). As expected, in our studies, overproduction of *EcoRRF* resulted in enhanced release of peptidyl-tRNA. We observed that overproduction of *EcoEFG* alone also resulted in increased accumulation of peptidyl-tRNA in

Table I. Plasmids and strains used

Plasmid/strain	Relevant details	Reference
pACDH	this plasmid harbours the ACYC origin of replication, and is derived from pACD (Mangroo and RajBhandary, 1995) vector by mutating the <i>Hind</i> III site of the Tet ^R marker to an <i>Nhe</i> I site	T.K.DineshKumar and U.Varshney (unpublished)
pACDH <i>Mtu</i> RRF	<i>M.tuberculosis</i> RRF cloned into pACDH	this work
pTrc <i>Eco</i> RRF	<i>E.coli</i> RRF cloned into pTrc99C	this work
pTrc <i>Mtu</i> RRF	<i>M.tuberculosis</i> RRF cloned into pTrc99C	this work
pTrc <i>Eco</i> EFG	<i>E.coli</i> EFG cloned into pTrc99C	this work
pTrc <i>Mtu</i> EFG	<i>M.tuberculosis</i> EFG cloned into pTrc99C	this work
<i>E.coli</i> AA7852	F ⁻ , <i>arg</i> ⁻ , <i>leu</i> ⁻ , <i>thr</i> ⁻ , <i>his</i> ⁻ , <i>thi</i> ⁻ , <i>pth</i> ^{ts}	Atherly and Menninger (1972)
<i>E.coli</i> LJ14	MC1061 containing the <i>frr</i> 14 ^{ts} allele	Janosi <i>et al.</i> (1998)
<i>E.coli</i> PEM100	<i>ara</i> Δ(<i>lac-proAB</i>) φ80 <i>lacZ</i> ΔM15 <i>fusA</i> 100 ^{ts}	Hou <i>et al.</i> (1994)
<i>E.coli</i> CA274	<i>HfrH</i> , <i>lacZam</i> , <i>trpEam</i>	Brenner and Beckwith (1965)

the *pth*^{ts} strain (data not shown) and inhibited the growth of *E.coli* AA7852 (*pth*^{ts}) (Figure 5B), suggesting that they work together in peptidyl-tRNA release. Although not reported so far, this observation predicts that another category of *pth*^{ts} suppressors could be the one where EFG expression is down-regulated. More importantly, as was the case at the post-termination step, overexpression of neither *Mtu*RRF nor *Mtu*EFG alone caused increased release of peptidyl-tRNA in the *pth*^{ts} strain. However, when the *Mtu*RRF and *Mtu*EFG were expressed simultaneously, enhanced release of peptidyl-tRNA was evident. Taken together, these observations suggest that peptidyl-tRNA release from the translating ribosome must lead to the dissociation of the translating ribosomes in much the same way as the post-termination complexes. Also, these studies suggest that RRF must compete with the aminoacyl-tRNAs for the occupancy of the ribosomal A-site. If the ribosomal A-site remains unfilled with an aminoacyl-tRNA for more than a 'threshold' period, these translating ribosomes are most probably destined for dissociation by RRF.

In conclusion, we have demonstrated that *E.coli* can be used as a surrogate host to investigate the mechanism of action of *M.tuberculosis* proteins. Since both RRF and EFG are essential proteins, development of this surrogate system is a major step to gain insights into the mechanistic aspects of RRF action in the cellular milieu. Notably, *M.tuberculosis* is an important pathogen that continues to cause most casualties worldwide. Thus, our use of the mycobacterial proteins in this study is also an important step forward in understanding the mechanism of action of the *M.tuberculosis* RRF which possibly can be used as an important drug target to control mycobacterial growth.

Materials and methods

Plasmids, strains and growth conditions

The various plasmids and strains used in this study are listed in Table I. LB liquid or solid (with 1.5% agar) media (Sambrook *et al.*, 1989) were used for growth. The medium was supplemented with various antibiotics at the following final concentrations: tetracycline 12.5 µg/ml and ampicillin 100 µg/ml, as required. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM when needed. The growth rate experiments were conducted at different temperatures by recording densities of fresh cultures at 600 nm at different times. Fresh cultures were started with 0.06% (v/v) inoculum from the overnight cultures grown at 30°C.

Cloning of RRF (*frr*) from *M.tuberculosis*

Based on the putative *frr* sequence of *M.tuberculosis* (Cole *et al.*, 1998), a forward (5'-GCGCCCATGGTTGATGAGGCTCTCTTC-3') and a reverse (5'-AGCGAAGCTTGAGCCTCCAGCAGCTC-3') primer containing *Nco*I and *Hind*III restriction sites, respectively, were designed to amplify the open reading frame (ORF) corresponding to RRF by PCR using chromosomal DNA from *M.tuberculosis*. PCR was performed using *Pfu* DNA polymerase (Promega) and the cycling conditions included an initial template denaturation step at 94°C for 5 min followed by 30 cycles of incubations at 94°C for 1 min, 45°C for 1 min and 68°C for 1 min. The PCR product of the expected size (555 bp) was obtained, digested with *Nco*I and *Hind*III and cloned into similarly digested pTrc99C. The authenticity of the clone was verified by DNA sequence analysis of the complete ORF. Subsequently, the RRF was also cloned into the pACDH vector between the *Nco*I and *Hind*III restriction sites.

Cloning of EFG (*fusA*) from *M.tuberculosis*

Using the available *fusA* sequence of *M.tuberculosis* (Cole *et al.*, 1998), a forward primer (5'-GCACAGAAGGACGTGCTGAC-3') starting from the second codon of the ORF and a reverse primer (5'-CTCGTAAGCTTGCGCTCACTC-3') which flanked the termination codon and contained a *Hind*III restriction site were designed. PCR was carried out using Vent DNA polymerase (*exo*⁺; New England Biolabs) and the cycling conditions consisted of an initial template denaturation step at 94°C for 5 min followed by 30 cycles of incubations at 94°C for 1 min, 58°C for 1 min and 68°C for 5 min. The PCR product was digested with *Hind*III, cloned into *Nco*I (blunted) and *Hind*III sites of pTrc99C vector and verified by DNA sequence analysis.

Cloning of the *E.coli* RRF gene (*frr*)

A forward (5'-CGTACCATGGGTAGCGATATCA-3') and a reverse (5'-GTCGCTGCAGAAATCAGAACTGC-3') primer containing *Nco*I and *Pst*I sites, respectively, were used to amplify the ORF corresponding to RRF (Ichikawa and Kaji, 1989) from *E.coli* genomic DNA using *Pfu* DNA polymerase. The reaction conditions were the same as detailed for the amplification of *Mtu*RRF. The PCR product was digested with *Nco*I and *Pst*I and cloned into similarly digested pTrc99C. Subsequently, *Eco*RRF was mobilized into pET11d using *Nco*I and *Hind*III restriction endonucleases.

Cloning of EFG from *E.coli*

PCR was done using *E.coli* CA274 genomic DNA and the *Eco*EFG gene (Zengel *et al.*, 1984)-specific forward (5'-ATGGCTCGT-ACA-ACACCCATC-3') and reverse (5'-GTATGGATCCTTAGGCTT-ATTTACC-3') primers using *Pfu* DNA polymerase. After an initial template denaturation step at 95°C for 3 min, 30 cycles of incubations at 95°C for 1 min, 55°C for 1 min and 70°C for 5 min were carried out. The PCR product was digested with *Bam*HI and cloned between end-filled *Nco*I (with the Klenow fragment of DNA polymerase I) and *Bam*HI sites of pTrc99C vector.

Purification of RRF and EFG from *E.coli* and *M.tuberculosis*

Details of the purification and biochemical characterization of these proteins will be described elsewhere. In brief, the respective ORFs were subcloned into the T7 RNA polymerase-based expression vector, pET11d, and the recombinants transformed into *E.coli* BL21 (DE3).

Fresh cultures of the transformants were induced with 0.5 mM IPTG, and the overproduced proteins were purified by gel filtration and ion exchange column chromatography. RRF proteins were purified to apparent homogeneity, whereas the EFG preparations contained a few detectable contaminating bands. Further characterization of the proteins was done by N-terminal sequence analysis and/or ESI MS.

Production of the anti-RRF polyclonal antibodies

Antibodies against RRF were raised in New Zealand rabbits by the standard method (Harlow and Lane, 1988) using Freund's incomplete adjuvant and purified on HiTrap protein G (Pharmacia Biotech) according to the manufacturer's protocol.

Preparation of cell-free extracts and immunoblotting

The cell-free extracts were prepared from log phase cultures (2 ml) of transformants exactly as described (Varshney *et al.*, 1991b) and quantified using Bradford's dye binding assay for protein estimation using bovine serum albumin (BSA) as a standard (Sedmak and Grossberg, 1977). Total cellular proteins (10 µg) were electrophoresed on SDS-PAGE (15%) and electroblotted (Towbin *et al.*, 1979) onto a nitrocellulose membrane (0.45 µm; Amersham) at 200 mA for 2 h. The membrane was blocked with 3% BSA in Tris-HCl-buffered saline (TBS; 20 mM Tris-HCl pH 7.5, 150 mM NaCl) for 2 h and then incubated for 1 h at room temperature with the purified anti-RRF antibodies (1:2000 dilution). After three washings with TBS, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Gibco-BRL) at a 1:2000 dilution for 1 h at room temperature. After three washings with TBS, the blot was developed with 12 µM *p*-nitroblue tetrazolium chloride and 6 µM 5-bromo-4-chloro-3-indolyl phosphate in 0.15 M Tris-HCl pH 9.5 containing 5 mM MgCl₂ to visualize the bands.

Preparation of total tRNA and northern blot analysis

Total tRNA was prepared under acidic conditions at 4°C, separated on acid urea gels and electroblotted onto nylon membranes exactly as described before (Varshney *et al.*, 1991a). The blot was probed with a 5'-³²P-labelled oligonucleotide complementary to tRNA^{Tyr} exactly as described before (Varshney *et al.*, 1991a) except that the SET buffer was substituted with the SSC buffer.

Polysome isolation

Polysomes were prepared according to the method described before (Tai and Davis, 1979) at 4°C. Briefly, *E.coli* MRE600 culture (1.5 l) was grown to log phase, supplemented with 0.3 mM tetracycline and quick-chilled on an ice-salt mixture. The cells were harvested by centrifugation at 5000 r.p.m. for 3 min using a GSA rotor (Sorvall), and suspended in 10 ml of buffer A (25% sucrose in 250 mM Tris-HCl pH 8.0). Subsequently, 4 ml of the lysozyme-Tris-Na₂EDTA solution (250 mM Tris-HCl, 8 mM Na₂EDTA pH 8.0 containing 40 mg of lysozyme) was added and, after keeping for 10 min, it was supplemented with 10 ml of a solution containing 1.67% Brij 35, 0.1% deoxycholate, 90 mM NH₄Cl, 35 mM magnesium acetate. After 5 min, 0.75 ml of 1 mg/ml DNase I was added and kept for 10–15 min. The lysate was centrifuged at 15 000 r.p.m. for 10 min using an SS34 rotor (Sorvall). The supernatant was stored and the pellet was resuspended in 5 ml of buffer B (10 mM Tris-HCl pH 7.8, 1 M NH₄Cl, 40 mM magnesium acetate, 10 mM 2-mercaptoethanol, 2 mM Na₂EDTA) and centrifuged at 15 000 r.p.m. for 10 min. The two supernatant fractions were pooled, layered onto a 60% sucrose solution in buffer B and subjected to ultracentrifugation for 8 h at 27 000 r.p.m. using an SW 28 rotor (Beckman). The pellet (polysomes) was suspended in RRF assay buffer and stored at -70°C.

In vitro assay for RRF activity

Various RRFs and EFGs were assayed using *E.coli* polysomes as substrates (Ohnishi *et al.*, 1999). The reaction mixtures (250 µl) containing polysomes (3.0 A₂₆₀) and various combinations of RRF/EFG were incubated at 32°C for 20 min in a buffer (10 mM Tris-HCl pH 7.4, 80 mM NH₄Cl, 8.2 mM MgSO₄, 1 mM dithiothreitol, 10 µM puromycin and 160 µM GTP) and fractionated on 15–30% sucrose gradients by centrifugation at 40 000 r.p.m. for 1 h using an SW50.1 Ti rotor (Beckman). The gradients were scanned from top to bottom at 254 nm using an ISCO gradient fraction collector.

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