Amber mutations in ribosome recycling factors of *Escherichia coli* and *Thermus thermophilus*: evidence for C-terminal modulator element

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Abstract Ribosome recycling factor, referred to as RRF, is essential for bacterial growth because of its activity of decomposition of the post-termination complex of the ribosome after release of polypeptides. In this study, we isolated a conditionally lethal amber mutation, named *frr-3*, in the *Escherichia coli* RRF gene at amino acid position 161, showing that the truncation of the C-terminal 25 amino acids of RRF is lethal to *E. coli*. An RRF gene cloned from *Thermus thermophilus*, whose protein is 44% identical and 68% similar to *E. coli* RRF, failed to complement the *frr-3*(Am) allele. However, truncation of the C-terminal five amino acids conferred intergeneric complementation activity on *T. thermophilus* RRF, demonstrating the modulator activity of the C-terminal tail. Rapid purification of *T. thermophilus* RRF was achieved by T7-RNA polymerase-driven overexpression for crystallography.

Key words: Ribosome recycling factor; *Thermus thermophilus* ribosome recycling factor; Amber mutation; Intergeneric complementation; Translation termination

1. Introduction

The termination of protein synthesis takes place on the ribosome as a response to a stop, rather than a sense, codon in the 'decoding' site (A site). Translation termination requires two classes of polypeptide release factors (RFs): a class I factor, codon-specific RFs (RF1 and RF2 in prokaryotes; eRF1 in eukaryotes), and a class II factor, non-specific RFs (RF3 in prokaryotes; eRF3 in eukaryotes) that bind guanine nucleotides and stimulate class I RF activity (review in [1]). After release of nascent polypeptides by RFs, the post-termination complex composed of the ribosome, deacylated tRNA and mRNA needs to be dissociated for the next round of protein synthesis [2]. It is likely that the ribosomal P site and A site remain occupied with a deacylated tRNA and a tRNA-mimicking RF protein (review in [3]), respectively, upon release of the polypeptide chain. A final step for recycling may be a direct decomposition of this complex; alternatively, a translocase may be required to forward deacylated tRNA and RF to the E and P sites of the ribosome, respectively, prior to the complete decomposition. In bacteria, the decomposition is catalyzed by a ribosome recycling factor (RRF, originally called ribosome releasing factor [4,5]) probably in concert with the elongation factor EF-G or RF3 in vitro (review in [1]). However, the mechanism of the RRF function is still very poorly understood.

Ehrenberg and his co-workers have reported that *Escherichia coli* RF3 accelerates the dissociation of the release factors RF1 and RF2 from the ribosome in a GTP-dependent manner and that fast recycling of ribosomes requires both RF3 and RRF [6,7]. Grentzmann et al. [8] have observed similar results and found that RF3 can substitute for EF-G in RRF-dependent ribosome recycling reactions in vitro. One contradiction that remains to be resolved is whether RRF is able to dissociate the mRNA or not. Pavlov et al. [7] have shown no release of the mRNA by RRF in vitro, and there are claims against the early work by Kaji and colleagues (review in [2]).

It is known that RRF is essential for bacterial growth since *E. coli* became lethal upon depletion of RRF [5] or by temperature-sensitive (Ts) mutations in RRF [9]. A reporter gene expression analysis suggests that inactivation of Ts RRF at the non-permissive temperature triggers unscheduled reinitiation of protein synthesis downstream of the stop codon, consistent with the predicted role for ribosome recycling, though the reinitiation mechanism remains to be understood in vitro. Perhaps for normal bacterial growth, more important is the need to recycle ribosomes without delay after synthesis of the protein product is completed. Recognition of a stop codon by RF is a slow process [10] and it is possible that a relatively high fraction of the ribosomal pool is bound to the stop codon. Rapid recycling of ribosomes should be of crucial importance for quickly growing bacteria. To date, RRF homologs are found universally in prokaryotes including a mitochondrial RRF of the budding yeast, *Saccharomyces cerevisiae*, but not in the eukaryotic cytoplasm. The latter observation, however, does not exclude the possibility that eukaryotes have an RRF-like activity in cytoplasm given that the recycling reaction is vital for growth.

In this study, we isolated a conditionally lethal amber mutation in the *frr* gene encoding the *E. coli* RRF (ecRRF), and cloned and analyzed the *Thermus thermophilus* RRF (ttRRF) gene by intergeneric complementation to initiate the systematic analysis of the structure-function relationships of RRF. Surprisingly, in spite of the inability of the intact ttRRF to complement the amber ecRRF mutation, ttRRF acquired intergeneric complementation capacity upon removal of the C-terminal five amino acids. This finding suggests that the C-terminal tail of RRF may have a modulator function. We will discuss this by clarifying the protein domains of RRF. Overexpression and mass purification of ttRRF are also presented for crystallography study.

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Abbreviations: RRF, ribosome recycling factor; ecRRF, *Escherichia coli* RRF; ttRRF, *Thermus thermophilus* RRF; RF, release factor; RF1, polypeptide release factor 1; RF2, polypeptide release factor 2; RF3, polypeptide release factor 3; Ts, temperature-sensitive; Am, amber; Km^R, kanamycin resistance; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Bacterial strains, plasmids and media

The *E. coli* K-12 strains and plasmids used are listed in Table 1. *T. thermophilus* (ATCC 27634) cells were grown at 70°C. The *E. coli* RRF coding sequence was amplified directly from strain W3110 by polymerase chain reaction (PCR) using primers 5′-GGGGGATCCA-TATGATTAGCGATATCAGA-3′ (primer 1, sense) and 5′-GGGG-GTCGACTCAGAACTGCATCAGTTC-3′ (primer 2, antisense), and cloned into plasmid pTWV228, giving rise to pTWV-ecRRF. Plasmid pTWV-ecRRFH6 is a derivative of pTWV-ecRRF, whose RRF gene was marked with a C-terminal histidine tag by PCR using the same sense primer and a His-tag encoding antisense primer (5′-GGG-GGTCGACTCAGTGGTGGTGGTGGTGGCTGAACTGCA-TCAGTTC-3′, primer 3). The sequence of the cloned RRF fragment was confirmed by DNA sequencing. The LB medium contained 1% Bacto-tryptone, 0.5% yeast extract and 0.5% NaCl [11].

2.2. Isolation of conditionally lethal amber mutants

P1 phage was first grown on strain MG12025 (*zad-220*::Tn*10*), and the phage lysate was incubated with 1 M hydroxylamine in 0.1 M potassium phosphate buffer (pH 6.0) at 37°C for 12 h. The mutagenized P1 phage was used to infect *E. coli* KH5402 (*supF6*[Ts]). Tetracycline-resistant (Tet^R) transductants were selected at 32°C, replica plated, and incubated at 42°C to screen for Ts mutants. These Ts colonies were characterized by suppression of amber alleles via lysogenization with transducing phages λ i^{21}pSuI (*supD*), λ pSuII (*supE*) and ϕ80 pSuIII (*supF*) as well as by complementation via transformation with plasmid pTWV-ecRRFH6. YN3576 (*frr-3*) is one of the RRF amber mutants thus isolated. The mutation was identified by DNA sequencing of DNA fragments amplified from the mutant chromosome by PCR using primer 1 and primer 2 or primer 3.

2.3. Preparation of a probe DNA

A pair of degenerated oligonucleotide primers, E_{UP} [5′-GT(C/G)-GA(C/G)-TAC-TAC-GG-3′ and E_{DOWN2} [5′-CG(C/G)-CG(C/G)-A(C/T)-GTT-(A/C)CG-(C/G)AC-3′], were used to amplify a 233-bp ttRRF sequence by PCR from *T. thermophilus* DNA. A pair of nested PCR primers, E_{UP} [5′-GT(C/G)-GA(C/G)-TAC-TAC-GG-3′ and E_{DOWN1} [5′-C(G/T)(A/G/T/C)-C(G/T)(C/T)-TC(C/T)-TC(A/G/T/C)-GT-(A/G/T/C)-A(A/G)-(A/G/T/C)G(C/G)-(A/G/T/C)GG-3′], were used to amplify a 206-bp nested fragment. This segment was directly used as a probe for Southern blot hybridization and plaque screening of the ttRRF gene.

2.4. Southern blotting

T. thermophilus DNA samples were digested with several endonucleases, and fragments were separated by 1% agarose-gel electrophoresis, transferred to Hybond N^+ filters (Amersham, Buckinghamshire, UK) and hybridized to the 206-bp PCR fragment probe. Detection of blot hybridization was performed by the non-radioisotope method using ECL Direct Nucleic Acid Labelling and Detection Systems (Amersham) according to the manufacturer's instructions.

2.5. Screening of T. thermophilus DNA library

A λEMBL3 phage [12] genomic library of *T. thermophilus* DNA was constructed using partial *Sau*3A fragments ranging in size over 7 kb (fractionated by agarose gel electrophoresis) by ligation to the *Bam*HI site. The library was screened with the 206-bp PCR product as described above, and phage λEMBL3-ttRRF was isolated. The DNA fragment cloned in λEMBL3-ttRRF was subcloned into plasmid pUC119 [13], giving rise to pUC-ttRRF.

2.6. Construction of overproducers of ttRRF and ecRRF

For overproduction of the ttRRF protein, the coding sequence in pUC-ttRRF was amplified by PCR using a sense primer (5′-(A/G/T/C)(A/G/T/C)(A/G/T/C)TCTAGAAATAATTTTGTTTAACTTTAAGA-AGGAGATATACATATGACCCTGAAGGAGCTTTACGCG-3′) and an antisense primer (5′-(A/G/T/C)(A/G/T/C)(A/G/T/C)GGATC-CTCAGCCCAGGATCTCCTGCTCCTT-3′). The amplified DNA contained the entire coding sequence of ttRRF. It was cloned downstream of a T7 RNA polymerase promoter between the *Xba*I and *Bam*HI sites of pET30a (Novagen, Madison, WI, USA) according to the manufacturer's instructions, giving rise to pET-ttRRF. The ttRRF gene was then marked with a C-terminal histidine tag by PCR using the same sense primer and a his-tag encoding antisense primer (5′-(A/G/T/C)(A/G/T/C)(A/G/T/C)GGATCCTCAGTGGTG-GTGGTGGTGGTGGCTGCCCAGGATCTCCTGCTCCTT-3′), and cloned into pET30a. The resulting plasmid pET-ttRRFH6 is structurally equivalent to pET-ttRRF. Plasmids overproducing ecRRF were constructed essentially by the same method as above except that *E. coli* RRF DNAs were amplified by PCR using primer 1 and primer 2 (for pET-ecRRF) or primer 3 (for pET-ecRRFH6).

2.7. Mutagenesis of plasmid-encoded frr and selection of suppressors

The pTWV-ttRRFH6 DNA was mutagenized by incubation with 0.4 M hydroxylamine at pH 6.0 for 20 h at 37°C. The plasmid was then precipitated with ethanol and rinsed several times with LB broth. The Ts lethal *frr* strain, YN3576 (*frr-3*), was transformed with the mutagenized DNA and temperature-resistant colonies were selected at 42°C on LB agar plates. Plasmid DNAs were recovered from these revertants, retransformed into the same parental strains, and those that gave a reproducible phenotype (i.e. growth at 42°C) were further characterized. One overcomer mutation in the plasmid-encoded RRF gene was isolated in a population of over 20 000 transformants.

2.8. Other DNA procedures

Single- or double-stranded DNAs were sequenced by means of dideoxynucleotide chain termination [14] using an ALF DNA sequencer (Pharmacia LKB). PCR proceeded according to standard methods [15] and other DNA manipulations were conducted according to standard methods [16].

3. Results

3.1. Isolation of an amber RRF mutation of E. coli

The *frr* gene maps at 4.0 min of the *E. coli* chromosome [17] and is close to one of the known transposon markers, *zad-220*::Tn*10*. Hence, *zad-220*::Tn*10* was used as a Tet^R selective marker for generating a conditionally lethal amber mutation in *frr* by transduction of hydroxylamine-treated (allowing both C→T and G→A substitutions) P1 phage to the parental strain KH5402. The KH5402 strain harbored the Ts amber suppressor allele, *supF6*(Ts), that incorporates tyrosine at the amber codon, UAG, at 32°C but not at 42°C [18], and hence an amber mutation in any essential gene is expected to show

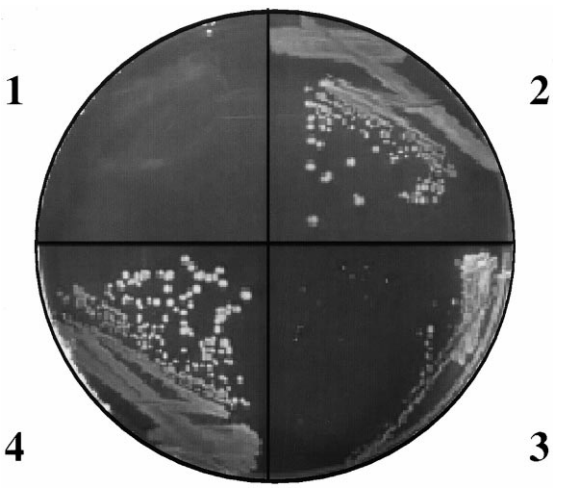

Fig. 1. Complementation of the *frr-3*(Am) mutation of *E. coli* by the *T. thermophilus* RRF gene. The Ts lethal *frr-3* strain (YN3576) was transformed with the respective plasmids and examined for growth phenotype. Transformant cells were streaked on an LB agar plate and incubated at 42°C. The following plasmids were used: sample 1, pTWV228 (vector control); sample 2, pTWV-ttRRF*181; sample 3, pTWV-ttRRFH6; sample 4, pTWV-ecRRFH6.

the Ts lethal phenotype [19]. YN3576 is one of the Ts Tet^R transductants thus isolated, whose growth was restored upon transformation with plasmid pTWV-ecRRFH6 that encodes only the RRF open reading frame within the insert DNA (Fig. 1, sample 4). The mutant also grew at 42°C upon lysogenization of transducing phages carrying the amber suppressors, *supD* (λ i^{21}pSuI), *supE* (λ pSuII) and *supF* (ϕ80 pSuIII), showing that the allele is an amber mutation and that the incorporation of not only Tyr but also Ser and Gln (at position 161 of RRF as described below) can restore the protein activity. Hence, the amber allele of YN3576 is designated *frr-3*(Am).

The *frr-3* DNA was amplified by PCR and sequenced. A single-base substitution of T for C was found at bp position 481 of the coding sequence, which substitutes the UAG stop codon for Gln-161, truncating the C-terminal 25 amino acids (Fig. 2).

Finally we confirmed the genetic map of the *frr-3*(Am) allele by P1 transduction. The first cross was conducted using the mutant YN3576 as a donor and the parental strain KH5402 as a recipient. The Ts lethality was cotransduced with *zad-220*::Tn*10* (five of 123) into the recipient that carries *supF6*(Ts). On the other hand, when the same P1 phage was infected to W3110 (*sup*^{0}), no Ts allele was transduced (0 of 100). The failure in transduction of Ts phenotype to W3110 indicates that RRF is essential for growth and the *frr-3* amber fragment is not active.

3.2. Cloning of an RRF gene from T. thermophilus

RRF sequences of different bacteria contain five highly conserved regions, I–V (see Fig. 3). Hence, three degenerated oligonucleotide primers, E_{UP} (region II), E_{DOWN1} (region V) and E_{DOWN2} (region IV) (see Section 2), were designed according to the sequences of the three most conserved regions which appear in the similarity plot and used for PCR amplification of a conserved DNA sequence. PCR by E_{UP} and E_{DOWN2} primers amplified a 233-bp segment from *T. thermophilus* DNA, and the nested PCR by E_{UP} and E_{DOWN1} manifested a 206-bp segment amplified from the 233-bp DNA segment (data not shown).

The 206-bp nested PCR product was used as a probe to screen for a λEMBL3 library of *T. thermophilus* DNA. Phage λEMBL3-ttRRF was one of the positive clones thus isolated, and the partial *Sau*3A insert was further digested by *Bam*HI. The 3-kb *Bam*HI fragment hybridizable to the 206-bp PCR probe was recloned to pUC119, giving rise to pUC-ttRRF.

The DNA insert recloned in pSU-ttRRF was sequenced (Fig. 2). The DNA and the deduced protein sequences were very similar to *E. coli frr* with the same number of amino acids. Its amino acid sequence is 44% identical (and 67% similar) to ecRRF, and the calculated molecular mass is 20 994 Da.

3.3. Intergeneric complementation of E. coli amber RRF mutant

The ttRRF gene cloned in pTWV-ttRRFH6 was tested for intergeneric complementation of the *E. coli frr-3*(Am) strain YN3576. However, pTWV-ttRRFH6 failed to restore growth of the *frr-3* mutant at 42°C under any experimental conditions tested (Fig. 1, sample 3). In spite of the apparent defect in complementation, tiny colonies appeared after prolonged incubation, suggesting that weak but significant residual activity may remain in ttRRF under the heterologous condition (data not shown).

We then explored whether any alteration(s) in ttRRF give rise to the activity of complementing the *frr-3* allele. To generate a plasmid-borne ttRRF mutation capable of suppressing the *frr-3* allele of *E. coli*, the pTWV-ttRRFH6 DNA was mutagenized in vitro with hydroxylamine and temperature-resistant revertants of YN3576 were selected directly upon transformation. Plasmid DNAs were isolated from these colonies and retransformed into the same YN3576 (*frr-3*) strain. After discarding those that failed to give a reproducible phenotype (i.e. growth at 42°C), we isolated a pTWV-ttRRFH6

Table 1
Bacterial strains and plasmids

Strains or plasmids	Relevant description	Source or reference
Escherichia coli K-12		
MG12025	*zad-220*::Tn*10*	National Institute of Genetics, Mishima
BL21 (DE3)	Lysogenic for λ DE3 carrying the gene for T7 RNA polymerase under the *lacUV5* promoter	Novagen
KH5402	*tyr*(Am) *trpE9829*(Am) *thr metE ilv thy supF6*(Ts)	[19]
YN3576	KH5402 *frr-3* (Am) *zad-220*::Tn*10*	This paper
W3110	Prototroph	Laboratory stock
Plasmids		
pUC119	*E. coli* cloning vector, Ap^R	[13]
pUC-ttRRF	*T. thermophilus* RRF coding segment cloned under P*lac* in pUC119, Ap^R	This work
pTWV228	*E. coli* cloning vector, Ap^R	TaKaRa
pTWV229	*E. coli* cloning vector, Ap^R	TaKaRa
pTWV-ecRRFH6	*E. coli* RRF coding segment cloned under P*lac* in pTWV228, Ap^R	This work
pTWV-ttRRFH6	*T. thermophilus* RRF coding segment cloned under P*lac* in pTWV229, Ap^R	This work
pTWV-ttRRF*181	Same as pTWV-ttRRFH6 but amber mutation for Gln-181	This work
pET30a	Multi-cloning-site expression vector under the control of T7 RNA polymerase promoter, Km^R	Novagen
pET30-ttRRF	*T. thermophilus* RRF coding segment cloned under T7 promoter in pET30a, Km^R	This work
pET30-ttRRFH6	Same as pET30-ttRRF but *T. thermophilus* RRF gene with a histidine tag, Km^R	This work
pET30-ecRRF	Same as pET30-ttRRF but *E. coli* RRF gene, Km^R	This work
pET30-ecRRFH6	Same as pET30-ttRRF but *E. coli* RRF gene with a histidine tag, Km^R	This work

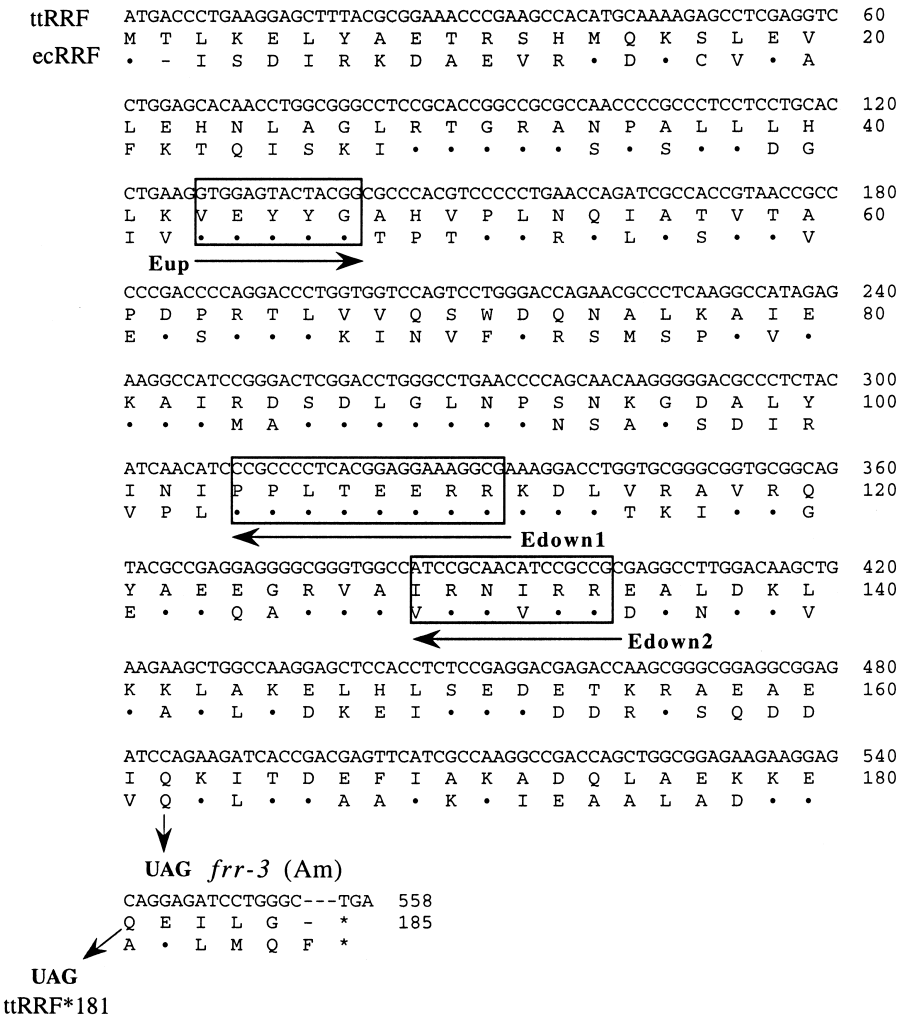

Fig. 2. Nucleotide and deduced amino acid sequences of the *T. thermophilus* RRF gene. Nucleotide and amino acid coordinates are counted from the initiation site of the *frr* coding sequence. Three boxed regions represent highly conserved motifs, which were used as primer sequences, E_{UP}, E_{DOWN1} and E_{DOWN2}, for PCR amplification. The *E. coli* RRF protein sequence is indicated under the *T. thermophilus* RRF sequence for comparison: only different amino acids are shown. Identical amino acids are represented by dots, and deletion of a base or an amino acid for optimal sequence alignment is shown by a hyphen. Two amber mutations, one (ttRRF*181) in ttRRF and the other (*frr-3*) in ecRRF, are noted. The nucleotide sequence data of the *T. thermophilus* RRF gene reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number AB016498.

derivative, referred to as pTWV-ttRRF*181, that restored the growth of YN3576 (Fig. 1, sample 2).

DNA sequencing revealed that the predicted ttRRF gene carried a single-base substitution of T for C at bp position 541 and substitutes the UAG stop codon for Gln-181 (hence designated ttRRF*181), removing the C-terminal five amino acids (see Fig. 2). These results strongly suggested that this protein-coding sequence comprises a functional ttRRF gene and that the C-terminal five amino acids of ttRRF are dispensable but affect the activity of intergeneric complementation over the *E. coli* RRF allele.

3.4. Overexpression and purification of T. thermophilus RRF

For overproduction of the ttRRF protein, the *frr* coding sequence was cloned downstream of a T7 RNA polymerase promoter (see Section 2), and the resulting plasmid pET30-ttRRF was transferred to BL21 (DE3). BL21 (DE3) contains a lysogenic lambda phage derivative, DE3, carrying the gene for T7 RNA polymerase under the control of an inducible *lacUV5* promoter. Expression of a recombinant protein cloned into the pET vector is driven by T7 RNA polymerase induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG). pET-ttRRF-bearing BL21 (DE3) cells were grown in the presence of 0.5 mM IPTG for 2.5 h, and total proteins were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 4 (lane 1), ttRRF was markedly overproduced under these expression conditions.

To achieve rapid purification of a recombinant RRF, a *T. thermophilus frr* gene was marked with a C-terminal histidine tag (see Section 2). The resulting plasmid pET-ttRRFH6 also overproduced ttRRF (Fig. 4, lane 5). The histidine-tagged ttRRF protein was then purified to homogeneity by affinity chromatography using ProBond resin (Invitrogen) followed by AKTA explorer 100 (Amersham Pharmacia Biotech) chromatography (Fig. 4, lanes 6 and 7). Purification is essentially based on the affinity of ProBond resin for tags consisting of consecutive histidine residues, and the process was rapid and suitable for mass purification. The above complementation

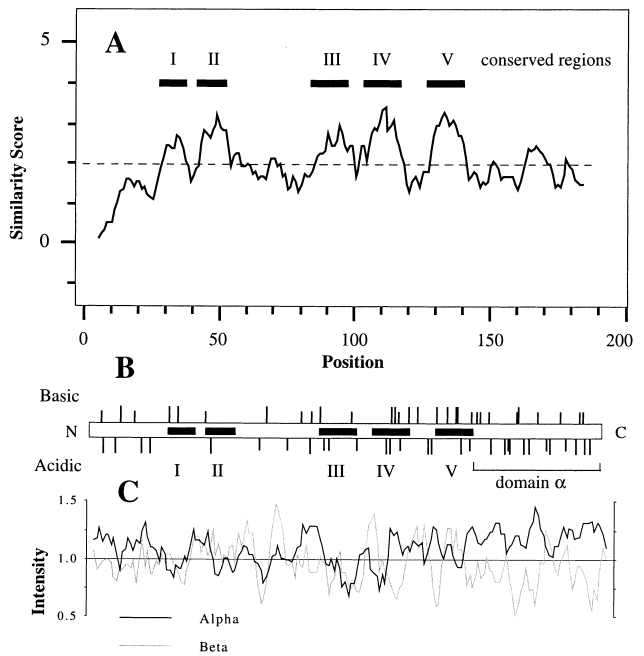

Fig. 3. The protein features of RRF. A: Average similarity plots of the 11 bacterial RRF sequences including: *Aquifex aeolicus* (accession number AE000703), *Bacillus subtilis* (accession number Z99112), *Brucella melitensis* (accession number U53133), *Chlamydia trachomatis* (accession number AE001338), *Escherichia coli* (accession number AE000126), *Haemophilus influenzae* (accession number U32763), *Mycobacterium leprae* (accession number Z97369), *Mycoplasma genitalium* (accession number U39730), *Staphylococcus aureus* (accession number AF033018), and *Helicobacter pylori* (accession number AE000631). Five conserved regions I–V are indicated by bold bars, three of which (i.e. regions II, IV and V) are used to design PCR primers. The average similarity score along the entire sequence is provided by the dashed line. Comparison scores (expressed in standard deviations) were calculated using the PILEUP program from the GCG program package [22]. B: Distribution of charged amino acids. Positions of acidic (Asp/Glu) and basic (Arg/Lys) amino acids in *T. thermophilus* RRF are shown by lower and upper vertical lines, respectively. Long (Glu/Arg) and short (Asp/Lys) bars represent the degree of acidicity or basicity. C: Secondary structure prediction. The plot shows the Chou and Fasman [23] propensity measures for α-helix and β-sheet calculated using the PepPlot program from the GCG program package [24]. The C-terminal part downstream of position 135 is referred to as domain α (see text).

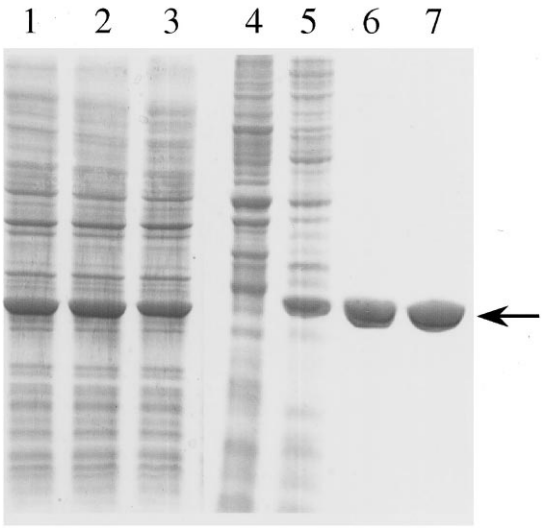

Fig. 4. Overproduction of *E. coli* and *T. thermophilus* RRF proteins. BL21 (DE3) cells carrying the respective plasmids were grown at 37°C in LB medium containing 50 μg/ml kanamycin, and log-phase cultures were exposed to 1 mM IPTG for 2.5 h. Bulk proteins were solubilized in 50 mM Tris-HCl (pH 6.8), 2% SDS and 2% β-mercaptoethanol by boiling for 3 min, separated by SDS-PAGE using 10% polyacrylamide gels, and stained with Coomassie brilliant blue. The protein determination was carried out by Bio-Rad Protein Assay (Bio-Rad Laboratories), and equal amounts of bulk proteins were loaded in lanes 1–3. The histidine-tagged ttRRF protein was purified to homogeneity by affinity chromatography using ProBond resin (Invitrogen) followed by AKTA explorer 100 (Amersham Pharmacia Biotech) chromatography using RESORCE column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Plasmids: lane 1, pET-ttRRF (bulk proteins); lane 2, pET-ecRRFH6 (bulk proteins); lane 3, pET-ecRRF (bulk proteins); lane 4, pET30a (control bulk proteins); lane 5, pET-ttRRFH6 (bulk proteins); lane 6, pET-ttRRFH6 (affinity-purified protein by ProBond resin); lane 7, pET-ttRRFH6 (chromatography-purified protein by RESORCE column). The arrow indicates the position of RRF.

data indicate that the C-terminal histidine tag does not affect RRF activity. Hence, the histidine-tagged RRF protein as well as the authentic RRF of *T. thermophilus* is being used for crystallography in this laboratory.

4. Discussion

In this study, we cloned the RRF gene from *T. thermophilus* and found that it has the potential activity to complement the RRF amber mutation, *frr-3*, of *E. coli*. The authentic ttRRF did not restore the growth of *frr-3*(Am) but, once the C-terminal five amino acids were deleted, it acquired the activity to rescue the lethality. This intergeneric complementation is very interesting in view of the evolutionary distance between *T. thermophilus* and *E. coli*. In fact, *T. thermophilus* RF1 does not complement the *E. coli* RF1 mutation in vivo regardless of having a weaker (than *E. coli* RF1) but significant activity to catalyze polypeptide release on the *E. coli* ribosome in vitro [20]. These results suggest that the essential structure-function domain(s) of RRF are highly conserved among Gram-positive and Gram-negative bacteria.

The RRF genes so far cloned from different organisms share five conserved peptide sequences designated, in this paper, regions I–V (see Fig. 3A). The relatively less stringent complementation activity of the heterologous RRF protein may be explained by assuming a conserved domain-domain interaction between RRF and the ribosomal complex. One could speculate that domains I–V of RRF may interact with conserved component(s) of ribosomal protein(s) or rRNA, or with mRNA.

One peculiar feature of the RRF structure is the localization of many charged amino acids in its C-terminal half (Fig. 3B). Of 78 amino acids downstream of position 107 of ttRRF, 19 are acidic (Asp and Glu) and 19 are basic (Arg and Lys) residues. This unusually high proportion of charged amino acids in the C-terminal region is also conserved in other RRF sequences. One could speculate that these charged residues form a structural unit separate from the N-terminal domain(s), or are localized on the surface of the N-terminal domain(s). The secondary structure prediction shows the high probability of α-helices at this region (hence designated

domain α; see Fig. 3C). Representation of residues 140–160 on a helical wheel suggests the presence of a potential amphipathic α-helix having (negatively and positively) charged residues along one face (data not shown). It is known that prokaryotic and eukaryotic transcription factors bear amphipathic helices with negatively charged residues along one surface and hydrophobic residues along another that are involved in the mechanism of transcription activation [21]. One might extend this analogy to RRF by assuming that the potential amphipathic α-helix in domain α is involved in activation of the decomposition of the post-termination ribosomal complex in spite of the different compositions of the charged residues in both helices, or in dimerization of RRF, though both scenarios are simply speculative yet. Nevertheless, the work described in this paper provides some evidence for the potential regulatory function of the C-terminal domain of RRF.

It should be noted first that the C-terminal four or five amino acids (except for *Bacillus subtilis* RRF due to its extension of 14 residues compared with others) are relatively less conserved (see Fig. 3). Because ttRRF gained intergeneric complementation capacity over the *E. coli frr-3* allele upon removal of this short C-terminal tail, we assume that this polypeptide region is capable of modulating the activity of ttRRF. It is tempting to speculate that domain α bears a potential activation function (as described above) and is modulated by the C-terminal tail. Given that ttRRF is a weak RRF compared with ecRRF, the C-terminal truncation may activate ttRRF. On the other hand, given that ttRRF is a strong, hence toxic, RRF in *E. coli*, this truncation may reduce the toxic activity.

Although these two possibilities remain to be investigated in vitro, we favor the scenario that the C-terminal tail of ttRRF is less accessible to the post-termination complex of *E. coli* ribosome due to species-specific variation(s) in the C-termini. It is noteworthy that, when the expression of ttRRF is controlled by the lactose promoter by different IPTG concentrations, no significant dose effect was observed on the complementation of the *frr-3*(Am) mutant under any experimental conditions tested (data not shown). Therefore, it seems less likely that the putative modulation by the C-terminal tail is simply to alter the strength of the protein activity, which could be compensated to some extent by the protein dosage. Rather, we assume that the C-terminal truncation gave rise to a species-non/less-specific RRF protein from ttRRF accessible to the *E. coli* post-termination complex.

Systematic deletion analyses of ecRRF revealed that it remains active in vivo by deletion of the C-terminal four amino acids, becomes conditionally (Ts) active by deletion of 5–10, and loses the activity by the further deletion (T. Fujiwara, K. Ito and Y. Nakamura, to be published elsewhere). Therefore, the C-terminal short tail is dispensable for cell growth but is capable of modulating the activity of RRF. It remains to be examined if the predicted modulator function is of physiological significance to control the ribosome recycling process in bacteria. Further studies are needed to clarify structure-function relationships of ribosome recycling factor domains.

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