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Evidence for *in vivo* ribosome recycling, the fourth step in protein biosynthesis

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Ribosome recycling factor (RRF) catalyzes the fourth step of protein synthesis *in vitro*: disassembly of the post-termination complex of ribosomes, mRNA and tRNA. We now report the first *in vivo* evidence of RRF function using 12 temperature-sensitive *Escherichia coli* mutants which we isolated in this study. At non-permissive temperatures, most of the ribosomes remain on mRNA, scan downstream from the termination codon, and re-initiate translation at various sites in all frames without the presence of an initiation codon. Re-initiation does not occur upstream from the termination codon nor beyond a downstream initiation signal. RRF inactivation was bacteriostatic in the growing phase and bactericidal during the transition between the stationary and growing phase, confirming the essential nature of the fourth step of protein synthesis *in vivo*.

Keywords: ribosomal frameshift/ribosome recycling/ temperature-sensitive RRF/termination complex/ translational re-initiation

Introduction

Protein biosynthesis consists of four steps: initiation, elongation, termination, and disassembly of the ribosome–mRNA–tRNA post termination complex (hereafter referred to as the termination complex). A survey of the current textbooks of biological sciences, however, shows that most authors complete the description of protein synthesis with termination, as if the fourth step of protein synthesis did not exist. Neglecting the fourth step of protein synthesis is a gross oversight. A recent review addresses this issue (Nakamura *et al.*, 1996).

Ribosome recycling factor (RRF)—formerly called ribosome releasing factor (see recent review by Janosi *et al.*, 1996a)—was identified as an *in vitro* protein synthesis factor as early as 1970 (Hirashima and Kaji, 1970). Since then, RRF, together with elongation factor G (EF-G), have been shown to catalyze the fourth step of protein synthesis *in vitro* (Hirashima and Kaji, 1972a,b, 1973; Ogawa and Kaji, 1975). The absence of RRF *in vitro* causes the terminating ribosomes to remain on mRNA and initiate protein synthesis at the codon immediately 3′ to the termination codon. In addition, RRF stimulates *in vitro* protein synthesis as much as 8-fold (Ryoji *et al.*, 1981b), suggesting that recycling the components of the termination complex increases the efficiency of protein synthesis *in vitro*. Our *in vitro* studies on RRF using natural mRNA (Ogawa and Kaji, 1975; Ryoji *et al.*, 1981b) were mostly confirmed recently by elegant *in vitro* studies with short synthetic mRNA (Pavlov *et al.*, 1997). Recent studies in our laboratory indicate that it may prevent errors *in vitro* during peptide elongation (Janosi *et al.*, 1996b).

Although there is no direct *in vivo* evidence available for ribosome recycling by RRF, there exists significant evidence that RRF is biologically important. The crucial biological role of RRF became evident when it was found that the gene encoding RRF is widely distributed in prokaryotes (Himmelreich *et al.*, 1996; Vizcaino *et al.*, 1996) including *Mycoplasma genitalium* (Fraser *et al.*, 1995). (For a complete list of other known RRF sequences, see Janosi *et al.*, 1996b.) *Mycoplasma genitalium* is the smallest known free-living organism with only ~500 genes; this organism has dispensed with other protein synthesis factors such as RF2 and RF3 which are involved in termination but has retained RRF. In *Escherichia coli*, RRF has been shown to be an essential factor for growth (Janosi *et al.*, 1994).

In this communication, we demonstrate *in vivo* evidence for RRF function using *E.coli* temperature-sensitive (Ts) mutants with altered RRF genes (*frr*). In agreement with previous *in vitro* results (Ryoji *et al.*, 1981a), translational re-initiation occurs downstream from the termination codon *in vivo* upon inactivation of RRF. The downstream re-initiation is at multiple random sites up to 45 nucleotides 3′ to the termination codon and does not require a canonical initiation codon sequence. This suggests that, in the absence of RRF, the ribosome of the termination complex remains on the mRNA, scans a short distance downstream, and re-initiates translation *in vivo*. Inactivation of RRF during the transition from the stationary to the growing phase had a bactericidal effect, while it had a bacteriostatic effect during the growing phase.

Results

Isolation of 12 independent alleles of frr coding for temperature-sensitive RRF

To prepare Ts RRF mutants, targeted random mutagenesis was performed on the wild-type RRF DNA (*frr*) by the error-prone PCR method. The mutagenized DNA was subcloned in a plasmid encoding CM^r (chloramphenicol resistance). Since this is a mixture of mutagenized *frr* DNA cloned into a plasmid, it was called pMIX. The

Table I. *Escherichia coli* strains and plasmids

Name	Genotype/relevant character[a]	Reference
Strains		
MC1061	F$^-$ *frr*(wild-type(Wt)) *araD139 (ara-leu)7679 (lacIPOZYA)X74 galU galK hsdR2 mcrB1 rpsL*(Sm^r)	Janosi *et al*., 1994
LJ3	MC1061 *frr1*[frame-shift(Fs)] Δ*(srl-recA)306*::Tn*10*. The *frr1*(Fs) allele does not encode functional RRF. For growth, the strain requires functional RRF which can be supplied by plasmid with *frr*(Wt).	This study
LJ3(pPEN1560)	LJ3 transformed with pPEN1560 plasmid. The plasmid carries *frr*(Wt) (required for growth) and the sacB-Neo^r cassette encoding sucrose sensitivity and Km^r	This study
LJ13	MC1061 *frr13* [temperature-sensitive (Ts)]	This study
LJ14	MC1061 *frr14* (Ts)	This study
LJ15	MC1061 *frr15* (Ts)	This study
LJ16	MC1061 *frr16* (Ts)	This study
DH5α	F$^-$ ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF)U169 deoR recA1 endA1 hsdR17 phoA supE44* λ$^-$ *thi1 gyrA96 relA1*	Gibco-BRL
Plasmids		
pUC19	Multicopy cloning and sequencing vector. Encodes AP^r	Yanisch-Perron *et al*., 1985
pSC101	Oligocopy naturally occurring plasmid. Encodes TC^r	ATCC
pMW119	Oligocopy cloning vector with pSC101 replicon. Encodes AP^r	Nippongene
pLKC480	pBR322-derived gene fusion reporter (β-galactosidase)vectors. Encodes KM^r and AP^r	Tiedeman and Smith, 1988
pLKC481	As pLKC480, but the multiple cloning site is two nucleotides shorter	Tiedeman and Smith, 1988
pMAK705	Oligocopy cloning vector with temperature-sensitive pSC101 replicon. Encodes CM^r	Hamilton *et al*., 1989
pIB209	pBR322-derived plasmid carrying the *sacB*-Neo^r cassette as a 3.8 kb *Bam*HI insert. Encodes KM^r and AP^r	Blomfield *et al.*, 1991
pRR1	pUC19 carrying a 2.2 kb *Eco*RI fragment from the *E.coli* chromosome which contains wild-type *frr*. Encodes AP^r	Ichikawa and Kaji, 1989
pRR2	pUC19 derivative carrying the *frr*(Wt) cistron with upstream and downstream flanking regions (including promoter and transcription termination sequences)	Shimizu and Kaji, 1991
pPEN728	pMW119 with *frr*(Wt) insert. Encodes AP^r	This study
pPEN874	Temperature-resistant (in replication) derivative of pMAK705. Encodes CM^r	This study
pPEN907	pPEN874 with *frr*(Wt) insert. Encodes CM^r	This study
pPEN1560	pPEN728 derivative which lost AP^r and carries the *sacB* (sucrose sensitivity)-Neo^r (KM^r) cassette	This study
pPEN2345	pLKC480 derivative with in-frame *lacZ′-′lacZ* gene fusion. Encodes AP^r and KM^r	This study
pPEN2363	pPEN2345 with a translational termination codon between *lacZ′* and *′lacZ. lacZ′-′lacZ* in-frame re-initiation reporter. Encodes AP^r and KM^r	This study
pPEN2367	pLKC481-derivative with out-of-frame *lacZ′-′lacZ* gene fusion	This study
pPEN2369	pPEN2367 with a translational termination codon between *lacZ′* and *′lacZ-′lacZ* out-of-frame re-initiation reporter. Encodes AP^r and KM^r	This study
pPEN2399	pUC19 with *frr14*(Ts) insert. Encodes AP^r.	This study
pAB93/pSM11	3A′ reporter vectors. Encode AP^r	Mottagui-Tabar *et al*., 1994
pSM–T272	Out-of-frame 3A′ re-initiation reporter plasmid. Encodes AP^r	This study
pSM–T275	In-frame 3A′ re-initiation reporter plasmid. Encodes AP^r	This study
pSEK336	pBR322-derived β-galactosidase reporter vector carrying the *orfA–orfB* junction region from insertion element IS*3*. Encodes AP^r	Sekine *et al*., 1994
pSEK336C	pSEK336 with inactivated *cl857* repressor gene. IS*3* re-initiation reporter. Encodes AP^r	This study

[a]Abbreviations: Fs, frame-shift; Ts, temperature sensitive; Wt, wild-type; AP^r, ampicillin resistance; CM^r, chloramphenicol resistance; TC^r, tetracycline resistance; KM^r, kanamycin resistance; Neo^r, neomycin-kanamycin resistance.

plasmids, pMIX, were then introduced into *recA*$^-$ *E.coli* LJ3(pPEN1560) which carries the functionally null mutant (frame shifted) *frr1*(Fs) in its chromosome (Janosi *et al.*, 1994). This *E.coli* harbors a resident plasmid, pPEN1560, containing wild-type *frr* [*frr*(Wt)] and the *sacB*-Neo^r cassette (conferring sucrose sensitivity and kanamycin resistance) (Blomfield *et al.*, 1991; see Table I). The resident plasmid (pPEN1560) and pMIX are incompatible because they have the same replicon. Growth at 32°C in the presence of sucrose and CM selects for segregants which lack the resident plasmid but harbor the plasmid with a mutagenized *frr* insert. Among 4548 transformants selected this way, 18 (0.4%) showed both the temperature- and the kanamycin-sensitive (because of loss of the resident plasmid) phenotypes. These transformants were considered to harbor a plasmid with a temperature-sensitive mutation in the *frr* gene.

To establish that the PCR-mutagenized *frr* is indeed responsible for the temperature sensitivity of the host, we isolated this portion from the plasmid of the 18 isolates and ligated it to plasmid pMW119 giving ampicillin resistance (AP^r). The *E.coli* strain LJ3 [*recA*$^-$ *frr1*(Fs)] harboring pPEN907 (incompatible with pMW119) which carries CM^r and wild-type *frr* was transformed with pMW119 carrying the *frr* (Ts) allele and Ap^r. From the AP^r transformants, CM-sensitive segregants which had lost their pPEN907 plasmid due to plasmid incompatibility were isolated. All of the isolated segregants were temperature- and CM-sensitive, establishing that the Ts phenotype is associated with the insert portion of the plasmids from our 18 Ts isolates.

To confirm that the Ts growth phenotype is associated with the open reading frame (ORF) of *frr*, the 0.9 kb *Kpn*I–*Hin*dIII fragment containing the promoter, the mutated

Table II. Mutational changes rendering the *E.coli* ribosome recycling factor temperature sensitive

Mutation				
Nucleotide change	Amino acid change	Notes	No. of isolates	Mutant allele
T(2)→C	Met1→Ala	N-terminal truncation	2	*frr8*
A(62)→G	Lys21→Arg	–	1	*frr15*
T(350)→A	Val117→Asp	–	2	*frr14*
T(383)→G	Val128→Gly	–	1	*frr2*
T(443)→C	Ile148→Thr	–	1	*frr6*
T(479)→A	Val160→Glu	–	1	*frr7*
T(488)→C	Leu163→Pro	–	3[a]	*frr4*
T(488)→C/G(475)→A	Leu163→Pro/Asp154→Asn	Double point mutation	1	*frr10*
G(535)→deletion	Glu174→Lys/Ala180→Gln Glu181→Asn/Leu182→Stop	Frameshift mutation and C-terminal truncation	1	*frr16*
G(541)→T	Glu181→Stop	C-terminal truncation. Also carries T(279)→A silent mutation	1	*frr13*
T(545)→C	Leu182→Pro	–	3	*frr17*
T(545)→C/C(26)→T	Leu182→Pro/Ala9→Val	Double point mutation	1	*frr3*

[a]One of the isolates also carries an A(–5) deletion between the ribosome binding site and the initiation codon.

frr, and the transcription terminator were isolated and sequenced. As shown in Table II, all mutational changes (sometimes more than a single change) fell within the coding sequence of *frr*. This suggests that the Ts growth phenotype is due to the mutational change in the coding sequence of *frr* carried by the plasmid.

Replacement of the chromosomal wild-type frr with its frr(Ts) allele and in vitro thermolability of the mutationally altered RRF

The chromosomal wild-type *frr* allele of the MC1061 strain was replaced with mutant alleles *frr13*, *frr14*, *frr15* and *frr16* via homologous recombination as described previously (Janosi *et al.*, 1994) resulting in strains LJ13 to LJ16 respectively. Strains LJ13 to 16 were all temperature-sensitive; furthermore, complementation of the Ts phenotype was observed with all of the strains by a plasmid carrying wild-type *frr* (data not shown). LJ14 was the most clear-cut Ts mutant.

To test the thermolability of RRF *in vitro*, *E.coli* LJ14 harboring pPEN2399 [pUC19 with *frr14*(Ts) insert], was grown at 32°C, and Ts RRF was purified to homogeneity. This preparation was then examined for its ability to convert polysomes into monosomes in the presence of EF-G and GTP (Hirashima and Kaji, 1970) after exposure to 45°C for varying periods. Figure 1 shows that the RRF coded for by *frr14* lost its ribosome-releasing activity upon exposure to the elevated temperature. Even with prolonged heat treatment, there was a residual (~20%) RRF activity. It should be noted that the specific activity of the purified Ts RRF was ~10% of that of the wild-type RRF even before the heat treatment (data not shown).

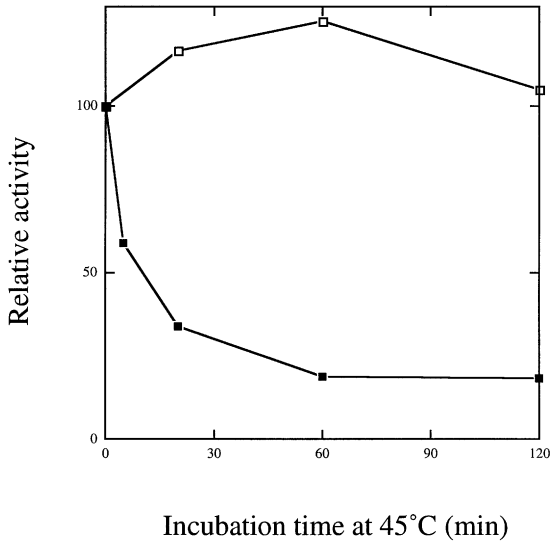

Fig. 1. Ribosome recycling factor coded for by *frr14* is temperature-sensitive. Wild-type RRF (□) and mutant RRF coded for by *frr14* (■) were purified to homogeneity (Hirashima and Kaji, 1972b). *In vitro* ribosome release assay (Hirashima and Kaji, 1972b) was carried out after exposing the isolated RRF to 45°C for varying periods as shown. Percentages of the activity remaining are shown.

Physiological characterization of the temperature-sensitive RRF mutants

Table III shows that the mutants fall into four major classes with regard to temperature sensitivity. The allele *frr8*, belonging to the first category, is moderately temperature-sensitive with only 90% reduction in the number of colonies at 43°C. With the most severely Ts mutants (*frr10, 14–16*), the colony-forming ability was reduced both at 37 and 43°C to 10^{-2} to 10^{-5} of that of wild-type.

Table III shows also that moving the mutant allele to the chromosome does not change the overall characteristics of the conferred Ts phenotype. The temperature sensitivity, however, increases when the mutant allele is moved to the chromosome. Since the oligocopy plasmid used in this test is present in approximately six copies (Hashimoto-Gotoh and Sekiguchi, 1977), this may be due to a dosage effect of the residual activity of Ts RRF, as indicated in Figure 1.

When the growing cells were exposed to the non-permissive temperature, the growth was reduced within 20 min (Figure 2, circles) but did not stop completely for a prolonged period, probably due to residual activity of Ts RRF at the non-permissive temperature. As shown in this figure (lower panel), when LJ14 [*frr14*(Ts)] cells were grown overnight at 32°C, diluted into fresh medium, and

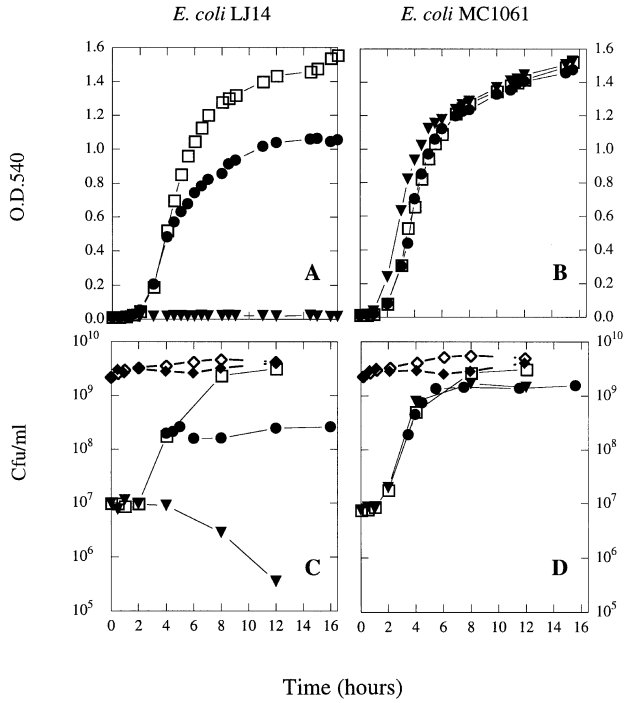

Fig. 2. Temperature-sensitive growth of *E.coli* carrying the mutant *frr14* allele in the chromosome. *E.coli* LJ14 [*frr14*(Ts)] (panels **A** and **C**) and its parent (wild-type *frr*) (panels **B** and **D**) were grown in Luvia–Bertani broth (LB) medium overnight at 32°C and divided into two parts. The first part was diluted with fresh LB to OD_{540} of 0.01 and divided into three aliquots. One aliquot of this diluted cell suspension was immediately exposed to 43°C and grown at that temperature (▼). The second aliquot was grown at 32°C until OD_{540} reached 0.45 when it was switched to 43°C (●). The third aliquot was grown at 32°C throughout the experiment (□). The second part from the overnight culture was further incubated at 32°C (◇) or at 43°C (◆) without dilution with fresh medium. The cell density was measured at various time points for the different culturing conditions and the results are plotted in panels A and B. In addition, aliquots from the different culturing conditions were removed at various times, plated, and grown at a permissive temperature. The number of viable colonies per ml is plotted in panels C and D.

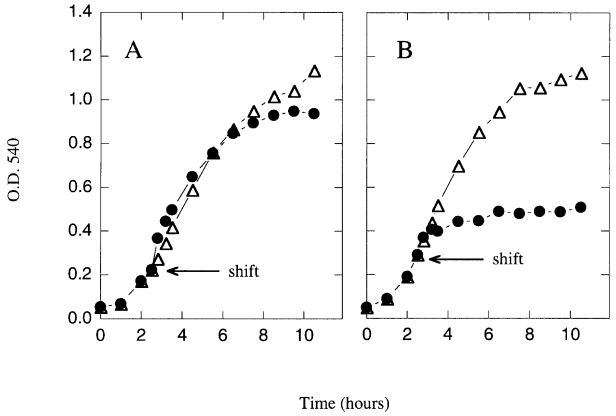

Fig. 3. Suppression of the temperature-sensitive growth phenotype by high gene dosage. *E.coli* LJ14 [(*frr14*(Ts)] harboring pPEN2399 [pUC19 plasmid containing *frr14*(Ts)] (**A**) and LJ14 harboring pUC19 (**B**) were grown at 27°C in LB supplemented with AP. The cultures were then diluted into the same medium to give 0.05 OD_{540} and grown at 27°C. At the time indicated by the arrows, the cultures were divided into two aliquots. One aliquot was kept at 27°C (△), and the other was shifted to 43°C (●).

exposed immediately to the non-permissive temperature, they started losing viability after 4 h at the high temperature (triangles). The loss of viability is dependent on the addition of fresh media (Figure 2, triangles versus closed diamonds). This suggests that the loss of RRF has a severe deleterious effect when cells switch from the stationary to the growing phase. Similar results were obtained with LJ13, LJ15 and LJ16 (data not shown).

In support of the notion that Ts RRF has the residual activity at the non-permissive temperature, we observed that an excess amount of the residual activity of Ts RRF expressed from multicopy pUC19 eliminated the Ts phenotype. Figure 3 shows that in such cells the effect of the non-permissive temperature was considerably delayed and diminished, compared with the control (panel B) which had only the chromosomal copy of the *frr14*(Ts) allele, suggesting a gene dosage effect. These results suggest that an excess Ts RRF does not exert toxic effects.

Amino acid alterations of RRF giving a temperature-sensitive phenotype

As shown in Table II, the 18 Ts isolates were represented by 12 independent genotypes in the 558 nucleotides encoding RRF (185 amino acids). In the N-terminal portion, we observed three mutations each involving the 1st, 9th and 21st codons respectively. In the *frr8* allele, the GUG initiation codon was changed to GCG (Ala) codon. A possible new translation initiation point at the Met13 codon probably functions in this case. At 5 nt upstream from Met13, there is an AAG which may function as a Shine–Dalgarno sequence for the 13th codon AUG. If this is the case, allele *frr8* has the most significant change in the primary sequence of RRF (loss of 12 N-terminal amino acids), yet it has only moderate thermolability (see Table III). Allele *frr3* is a double point mutant, with Leu182→Pro and Ala9→Val. This mutant is only slightly more temperature-sensitive than *frr17* with a single Leu182→Pro mutation, indicating that the mutation Ala9→Val has very little influence on thermostability. In contrast, the Lys21→Arg mutation (*frr15*) gives temperature sensitivity, even at 37°C. These three mutants therefore, suggest that at least 12 N-terminal amino acids are not important for RRF thermostability, but the maintenance of the sequence from somewhere beyond the 12th amino acid is important for thermostability, as indicated by the temperature sensitivity of *frr15* with the mutation at the 21st position.

In a further downstream region, between Lys21 and Val117 codons, mutations did not occur, suggesting that slight alterations in this region of the protein may be lethal or have no effect on thermostability. These possibilities are strengthened by the fact that there was more than one independent isolate with identical mutational sites in other regions.

In the portion further downstream from this large silent region, Ts mutations occurred approximately every 15 codons at positions 128, 148, 163 (two more nearby) and 179 (five more nearby at the C-terminal end). Mutations in the proximal and distal portion of the region stretching from amino acid 117 to 163 render the molecule labile, even at 37°C. Thus, *frr14* (Val117→Asp), *frr2* (Val128→Gly), *frr4* (Leu163→Pro) and *frr10* (Leu163→Pro and Asp159→Asn) are severely temperature-sensitive

Table III. Diversity of the temperature-sensitive growth phenotype determined by the mutant *frr* alleles

Mutant allele	Efficiency of plating[a]					
	Mutant allele in oligocopy plasmid			Mutant allele in the chromosome		
	32°C	37°C	43°C	32°C	37°C	43°C
frr8	1	1	10^{-1}	–	–	–
frr2	1	10^{-3}	10^{-3}	–	–	–
frr4	1	10^{-3}	10^{-3}	–	–	–
frr6	1	1	10^{-4}	–	–	–
frr7	1	1	10^{-4}	–	–	–
frr13	1	1	10^{-4}	1	1	$<10^{-5}$
frr17	1	1	10^{-4}	–	–	–
frr3	1	1	10^{-5}	–	–	–
frr15	1	10^{-2}	10^{-4}	1	10^{-4}	$<10^{-5}$
frr14	1	10^{-3}	$<10^{-5}$	1	10^{-4}	$<10^{-5}$
frr10	1	10^{-3}	$<10^{-5}$	–	–	–
frr16	1	10^{-2}	10^{-4}	1	10^{-4}	$<10^{-5}$

[a]The values represent the relative efficiency of plating of mutants to that of wild-type at the temperature indicated, using the semi-quantitative method described in Materials and methods. – indicates that we did not create strains which contain these mutant alleles in the chromosome.

(Table III). Mutations occurring in the middle of this region, as in *frr6* (Ile148→Thr) and *frr7* (Val160→Glu), render the molecule inactive only at 43°C. These data suggest that the ends of this stretch of 46 amino acids play a critical role in the thermal stabilization of RRF.

The two alleles, *frr13* and *frr16*, code for a C-terminal truncation, five and four amino acids long respectively. Allele *frr16* is a G(535) deletion mutant. It causes a frameshift, effecting codons 179 to 181 giving termination at codon 182. The *frr17* and *frr3* carry a common point mutation (Leu182→Pro) and exhibit approximately the same temperature-sensitive phenotype (inactivated at 43°C but not at 37°C) as *frr13* (loss of C-terminal amino acids 182 to 185). This suggests a critical role of Leu182 for stability. These C-terminal mutations also suggest that extensive alterations in the C-terminal region are not fatal. This region, however, must be important for the thermal stability of RRF because C-terminal mutants are all sensitive at 43°C and RRF encoded for by *frr16* is temperature-sensitive, even at 37°C. Consistent with this concept is the fact that the C-terminal end of RRF is well-preserved among RRF of various species (Janosi *et al.*, 1996a).

RRF functions to disassemble the termination complex in vivo

Earlier *in vitro* experiments (Ryoji *et al.*, 1981a) showed that translation in the absence of RRF results in re-initiation and translation downstream from the termination codon without depending on the initiation signals because ribosomes are not released from the termination complex in the absence of RRF (Ogawa and Kaji, 1975). To demonstrate that RRF releases ribosomes from the termination complex *in vivo*, we constructed two plasmids with a reporter gene coding for β-galactosidase to detect unscheduled translation downstream from the termination codon. In these constructs, the *lac* promoter is followed by the *lacZ* ribosome binding site (RBS) and a short ORF coding for 13 amino acids followed by a stop codon. The stop codon is followed by the *lacZ* coding sequence (from the 10th authentic *lacZ*) either in zero frame (pPEN2363) or in –2 frame (pPEN2369). The reporter *lacZ* lacks its own initiation codon.

The above-mentioned plasmids, pPEN2363 and pPEN2369 were placed separately into LJ14 [*frr14*(Ts)]. If ribosomes are not released due to the inactivation of RRF at 39°C, and they re-initiate translation downstream from the termination codon without depending on the initiation signals as we observed *in vitro*, one would expect the *lacZ* gene to be expressed. The controls were the wild-type parent strain (MC1061) or LJ14 harboring pPEN907, the plasmid encoding wild-type RRF.

Figure 4 shows that a temperature increase induced synthesis of β-galactosidase in LJ14 (Figure 4A, upper left panel) with the in-frame *lacZ′-′lacZ* reporter plasmid. The induction was minimal at 31°C, where most of the Ts RRF should be active. The induced β-galactosidase synthesis is mostly attributable to RRF depletion at 39°C because the MC1061 strain with wild-type RRF (Figure 4A, upper middle panel) and LJ14 harboring pPEN907 carrying wild-type *frr* (upper right panel) showed only a negligible β-galactosidase activity.

As shown in Figure 4B, when RRF was depleted, β-galactosidase was also synthesized in the cells transformed with pPEN2369, the out-of-frame (–2 frame) reporter plasmid (Figure 4B, upper left panel). If RRF was not inactivated, or wild-type RRF was present, no β-galactosidase synthesis was observed. As shown in Figure 5, the optimum re-initiation for the in-frame construct was at 35°C, while it was at 39°C with the out-of-frame construct. The observed difference suggests that ribosomes remaining at the termination codon in the post-termination complex may undergo thermal agitation to change the reading frame *in vivo*.

As controls, similar constructs without the upstream termination codons were prepared; pPEN2345 (with the *lacZ* gene in-frame with the initiation codon) and pPEN2367 (with the *lacZ* gene in –2 frame with the initiation codon). As expected, pPEN2345 constitutively expressed β-galactosidase in all situations, while

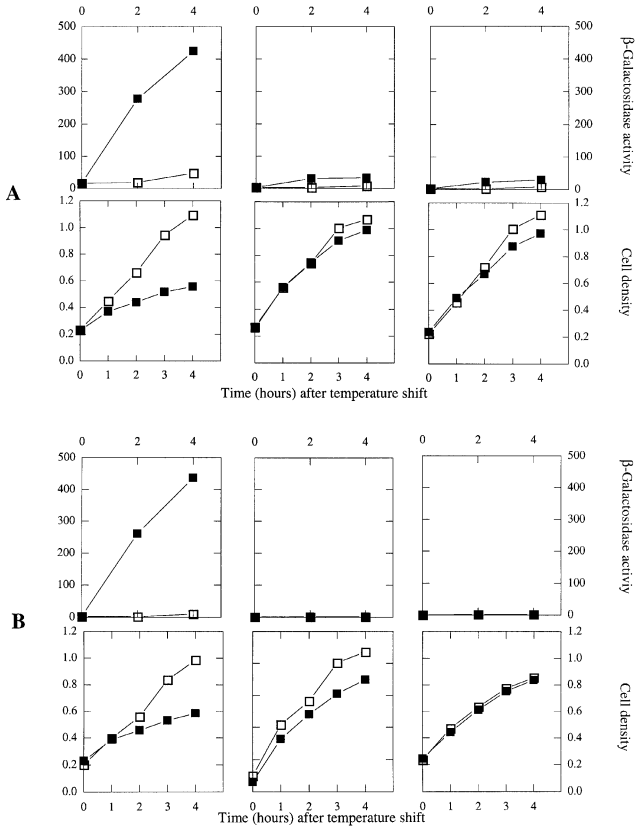

Fig. 4. Temperature-dependent re-initiation of translation downstream from the termination codon in LJ14 (Ts *frr*). Plasmid pPEN2363 (the β-galactosidase coding sequence is in-frame with the upstream termination codon) (**A**) or plasmid pPEN2369 (the β-galactosidase coding sequence is out-of-frame with the upstream termination codon) (**B**) was placed in *E.coli* LJ14 (left figures), or in its parent MC1061 (middle figures) or in LJ14 harboring pPEN907 carrying wild-type *frr* (right figures). The strains were grown in LB (supplemented with kanamycin) at 31°C. Then cultivation continued either at 31°C (□) or at 39°C (■) for an additional 4 h. β-Galactosidase activity (top row figures in both panels) and OD_{600} [bottom row figures in (A) and (B)] were measured as indicated. Data for □ at various time periods are overlapping with ■ in some of the figures of (B) and shown as ■.

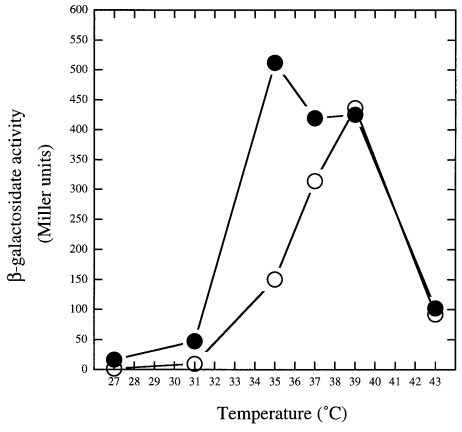

Fig. 5. Temperature optimum for out-of-frame re-initiation is higher than for in-frame re-initiation. *E.coli* LJ14 harboring either pPEN2363 (in-frame β-galactosidase reporter plasmid) (●) or pPEN2369 (out-of-frame β-galactosidase reporter plasmid) (○) was grown at 31°C and the cultures were shifted to the indicated temperatures and grown for another 4 h. The induced β-galactosidase activity was then measured and plotted against the temperature.

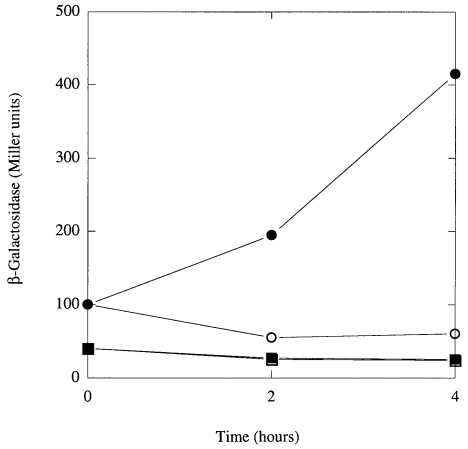

Fig. 6. Out-of-frame (–1) re-initiation of translation downstream from the termination codon of *orfA* of IS*3*. *E.coli* LJ14 [*frr14*(Ts)] (circles) and its parent MC1061[*frr*(Wt)] (squares) were transformed with pSEK336C which contains the reporter gene in the –1 frame with respect to the upstream termination codon. Transformants were grown in LB overnight at 32°C, diluted with the same medium, grown to 0.4 OD_{540}, and then one aliquot was further grown at 32°C (○,□), and the other was shifted to 39°C (●,■). The data representing ■ and □ are overlapping and shown with the ■. The β-galactosidase activity (in Miller units) is plotted against the periods after the temperature shift.

pPEN2367 did not express β-galactosidase in either the wild-type *E.coli* or in the *frr*(Ts)LJ14 mutant, regardless of the temperature (data not shown).

Since pPEN2369 used in the experiment shown in Figure 4 has the reporter in –2 frame, we wished to establish that –1 out of frame re-initiation of translation can also occur due to inactivation of RRF. We used plasmid pSEK336C which contains an upstream ORF (*orfA* of insertion sequence IS*3*) followed by the *orfB′-lacZ* fusion in –1 frame with respect to the termination codon of *orfA* (Sekine *et al.*, 1994). The initiator AUG codon of *orfB* was eliminated. In this construct, β-galactosidase is expected to be synthesized only by ribosomes that have finished the translation of *orfA* and re-initiate downstream out-of-frame from the *orfA* termination codon. Plasmid pSEK336C was placed in LJ14 [*frr* (Ts)], and the culture temperature was raised to 39°C. As shown in Figure 6, *in vivo* inactivation of RRF significantly increased β-galactosidase synthesis. At the permissive temperature for LJ14, or at both temperatures in the case of MC1061 (wild-type *frr*), such an increase was not observed.

The efficiency and the site of re-initiation caused by RRF inactivation in vivo

In the system described in the preceding section, only the re-initiated proteins with the reporter β-galactosidase, but not the translation of the upstream ORF, could be measured. It was therefore not possible to estimate the fraction of ribosomes that reach the upstream termination codon and engage in re-initiation as a result of RRF inactivation. To estimate this fraction, we used a modified version of the 3A′ reporter system (Björnsson *et al.*, 1996) in which we can quantify both the proteins that correspond to the upstream cistron and to the unscheduled re-initiated translation downstream from the termination codon. This system consists of an engineered protein A of *Staphylo-*

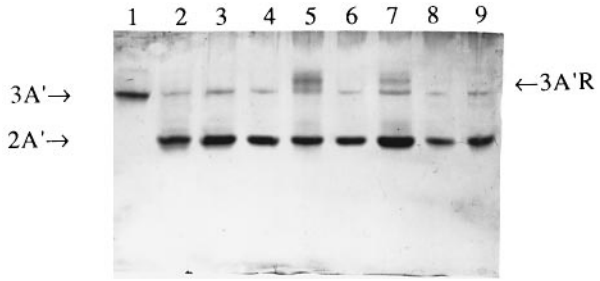

Fig. 7. Re-initiation of translation upon *in vivo* inactivation of RRF in *E.coli* LJ14 with the 2A′–3A′ system. *E.coli* MC1061 (wild-type *frr*) and its temperature-sensitive derivative *E.coli* LJ14 [*frr14*(Ts)] were transformed either with pSM–T272 (2A′ followed by 3A′ out-of-frame), or with pSM–T275 (2A′ followed by 3A′ in-frame). The transformants were grown overnight at 32°C in LB, diluted with fresh LB and grown at 32°C until OD_{540} reached 0.65. One aliquot was then grown at 32°C, and the other at 39°C for 1 h. IPTG (0.4 mM) was then added to induce expression of 2A′ and 3A′ for 1 h. The cultures were then treated as described (Björnsson *et al.*, 1996). Lane 1, 3A′ size control, MC1061/pAB93 with no stop codon insert (Mottagui-Tabar *et al.*, 1994) grown at 32°C; lane 2, MC1061/pSM–T272 grown at 32°C; lane 3, MC1061/pSM–T272 at 39°C; lane 4, LJ14/pSM–T272 at 32°C; lane 5, LJ14/pSM–T272 at 39°C; lane 6, LJ14/pSM–T275 at 32°C; lane 7, LJ14/pSM–T275 at 39°C; lane 8, MC1061/pSM–T275 at 32°C; lane 9, MC1061/pSM–T275 at 39°C. Arrows on the left indicate the 2A′ and 3A′ proteins. The arrow on the right marked as 3A′R indicates re-initiated proteins (in lanes 5 and 7).

coccus aureus [called A′ (Björnsson *et al.*, 1996)]. We made a construct so that the upstream cistron is a dimer of A′ (M_r =12 000; 2A′) and the downstream ORF is a trimer of protein A′ (M_r = 18 000; 3A′). The ATG initiation signal for 3A′ is preceded by a weak Shine–Dalgarno sequence giving only a minimal background initiation. Unscheduled re-initiated protein should be detected as proteins slightly larger than 3A′ due to the addition of amino acids at the N-terminal end coded by codons downstream from the 2A′ termination signal and extended translation into the 3A′ cistron.

Plasmid pSM–T272 (the downstream 3A′ sequence is in –1 frame with respect to the upstream 2A′ termination codon) and pSM–T275 (in zero frame) were placed separately in LJ14. As shown in Figure 7, all the test strains (lanes 2–9) synthesized 2A′ and 3A′ proteins regardless of the presence of RRF, as expected. Upon inactivation of RRF *in vivo* by raising the culture temperature to 39°C, proteins with slightly higher molecular weight than 3A′ were observed (bands 3A′R in lanes 5 and 7). In accordance with the *in vivo* re-initiation observed in Figures 4 and 6, 3A′R was observed with pSM–T272 (out-of-frame) as well as with pSM–275 (in-frame). No product smaller than 3A′ was observed as a result of RRF inactivation, suggesting that the ribosomes remaining on the mRNA cannot re-initiate downstream from the canonical initiation site of 3A′. This observation also suggests that non-specific proteolytic degradation of the re-initiated products is not significant in LJ14. Because 3A′R bands did not appear either in MC1061 at the elevated temperature (Figure 7, lanes 3 and 9) or in LJ14 at low temperature (lanes 4 and 6), we conclude that their appearance is due to inactivation of RRF by the elevated temperature.

The results suggest that ribosomes which are not released from the termination codon of 2A′ re-initiate upstream from the initiation codon of 3A′. This re-initiation must occur at several points, because the 3A′R band is not discrete in the gels. The results in Table IV show that the mean molar amount relative to that of 2A′ for the out-of-frame and the in-frame 3A′R products were 0.41 and 0.19, respectively. It should be pointed out that the system provides measurement only for the frame which gives 3A′ and 3A′R but not for the other two frames, which yield proteins that are eliminated in the purification of 2A′, 3A′ and 3A′R. The actual efficiency of re-initiation should therefore be higher than the values shown in Table IV. This then suggests that most, if not all, of the ribosomes terminating 2A′ may remain on the mRNA and begin the unscheduled translation in the absence of RRF.

Re-initiation in the absence of RRF occurs at several points and does not require canonical initiation codon

The results using the 3A′ system suggest that the proteins synthesized via re-initiation upon inactivation of RRF consist of a mixture of proteins having various N-termini depending on the site of re-initiation. As shown in Table V, this notion was confirmed by N-terminal analysis of β-galactosidase synthesized in the absence of RRF. In this experiment, the induced reporter β-galactosidase proteins from the experiments described in Figures 4 and 6 were purified and Edman analysis was performed on the mixture of the induced proteins. The results were compared with the DNA sequences of the plasmid constructs. Several different major peptides were detected as shown in Table V. The control analysis of β-galactosidase coded for by pPEN2345 (′*lacZ* coding sequence aligned in-frame with the initiation codon of the *lacZ*′ sequence without the upstream termination codon) gave the single N-terminal amino acid sequence encoded by *lacZ*′ and pUC19 (Fowler and Zabin, 1977; Yanisch-Perron *et al.*, 1985) (Table V; pPEN2345).

We could not detect any β-galactosidase which was initiated upstream from the termination codon. With pPEN2363 and pPEN2369, re-initiation can occur as far downstream as 22 and 23 nt from the termination codon respectively. With pSEK336C, re-initiation occurred as far as 45 nt downstream. None of the re-initiation points corresponds to the codon immediately downstream from the termination codon as was observed in the *in vitro* system (Ryoji *et al.*, 1981a). Although the possibility exists that these proteins are degradation products of a re-initiated protein, this is unlikely because we did not observe any re-initiated product smaller than 3A′ in the experiment described in Figure 7. If significant non-specific protein degradation occurs, one would expect smaller products than 3A′.

Discussion

On the basis of the following considerations, we propose that RRF does indeed catalyze the fourth step of protein biosynthesis, disassembly of the termination complex *in vivo*. First, *in vitro* data have established that RRF releases ribosomes at the termination codon on natural mRNA (Ogawa and Kaji, 1975) and on synthetic mRNA (AUG XXX UAA) (G.Grentzmann, P.J.Kelly, S.Laalami,

Table IV. Majority of ribosomes at the termination complex engage in translation downstream from the termination codon

Plasmid	Context	Expression of 3A′R[a]			
		MC1061		LJ14	
		32°C	39°C	32°C	39°C
pSM–T272	out-of-frame	0.014–0.022	0.021–0.034	0.018–0.026	0.188–0.573
pSM–T275	in-frame	0.023–0.050	0.006–0.046	0.017–0.050	0.150–0.230

[a]Gels, similar to that shown in Figure 7 were scanned and analyzed with NIH Image software (V.1.58) for band densities. Expression of 3A′R is given as 3A′R/2A′ ratio obtained by scanning of gels and correction for size (Björnsson and Isaksson, 1993).

Table V. N-terminal amino acid sequence of re-initiation proteins

Reporter vector	Partial nucleotide sequence[a]	Detected N-terminal sequence	Distance of reinitiation point from termination codon (nt)
pPEN2363 in-frame *lacZ′-′lacZ*	5′...**AGGCTCTAG**CTAGAGGATCCGTCGACCTGCAGCCAAGCTTGCGATCCCGTC...3′ L E D P S T C S Q A C D P V + + +	DPSTCS TCSQAC	7 16 22
pPEN2369 out-of-frame *lacZ′-′lacZ*	5′...**AGGCTCTAG**CTAGAGGATCCGTCGACCTGCAGCCAAGCTTGCTCCCGTC...3′ R I R R P A A K L A P V + + +	PAAKLA AAKLAP AKLAPV	17 20 23
pPEN2345 in-frame no termination	5′ - **ATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCG**...3′ M T M I T P S L H A C R S +	TMITP	O[a]
pSEK336C out-of-frame IS*3* reporter	5′...**CTGAACTGA**AGTATGTCTTTATTGAAAAACATCAGGCTGAGTTC Y V F I E K H Q A E F + + + + + +	FIEKHQ EKHQAE HQAEFS	9 15 21
	AGCATCAAAGATCCCGTCGTTTTACAACGTCGT...3′ S I K D P V V L Q R R + +	AEFSIK EFSIKD SIKDPV DPVVLQ	27 30 36 45

[a]The junction region between promoter proximal open reading frame (bold typeface characters) and the fused nucleotide sequence (plain typeface characters) is shown in 5′ → 3′ direction. Beneath the nucleotide sequence the translation of codons which are in-frame with the *lacZ* coding sequence is also shown (in one-letter codes for the amino acids). A + underneath the amino acid indicates translational re-initiation site.

M.Shuda, M.A.Firpo, Y.Cenatiempo and A.Kaji, manuscript in preparation). Recent *in vitro* experiments with short synthetic mRNA containing the ribosome-binding sequence suggest that released ribosomes may immediately bind to the mRNA again when the Shine–Dalgarno sequence is very close to the termination codon (Pavlov *et al.*, 1997). This would give the appearance of ribosomes not released from mRNA and slide back to the Shine–Dalgarno sequence on the mRNA. Second, in the absence of RRF, the ribosomes remaining at the termination codon on mRNA re-initiated translation starting at the codon immediately following the termination codon *in vitro* (Ryoji *et al.*, 1981a). Third, as shown in this paper, inactivation of RRF induced most, if not all, of the ribosomes to re-initiate unscheduled protein synthesis downstream from the termination codon in three separate *in vivo* systems.

Some features of *in vivo* translational re-initiation in the absence of RRF have been delineated by the current study. First, the re-initiation of protein synthesis *in vivo* occurred at multiple sites as far as 45 nt downstream from the termination codon (Table V). We suspect that, in the absence of RRF, the ribosome of the termination complex scans downstream from the termination codon without synthesizing protein. Eventually, ribosomes re-initiate protein synthesis without depending on a canonical start codon or a Shine–Dalgarno sequence. Second, frame shifting occurs during re-initiation; hence, the reporter protein was synthesized in the absence of RRF regardless of whether the reporter gene was in-frame (Figures 4 and 7), –2 nucleotides out-of-frame (Figure 4), or –1 nucleotide out-of-frame (Figures 6 and 7). This frame shifting was stimulated by elevated temperatures (Figure 5). To the best of our knowledge, RRF is the first reported soluble protein factor involved in preventing ribosomal frame shifting besides EF-G which functions to translocate ribosomes precisely three codons without causing frame shifting (Dahlfors and Kurland, 1990). Third, the translation re-initiation following inactivation of RRF occurs only downstream from the termination codon; therefore, no reporter protein which initiated upstream from the termination codon was detected (Table V). It should be noted however, that if re-initiation occurred upstream from the termination codon, only the out-of-frame reporter protein would be detected since the in-frame reporter protein would have an in-frame termination codon interrupting the sequence. Fourth, ribosomes which are scanning downstream from the termination site do not scan beyond an authentic initiation signal with a ribosome-binding sequence. Thus, in the 3A′ experiment described

in Figure 7, we did not detect any re-initiation products which were smaller than 3A′.

Inactivation of RRF was bacteriostatic in the growing phase. Like RRF, *in vivo* inactivation of any one of the currently known 10 essential protein synthesis factors causes bacteriostasis. The bacteriostatic effects of *in vivo* inactivation of these factors were demonstrated either with the use of temperature-sensitive mutants such as IF3 (Grunberg-Manago, 1996), EF-G (Hou *et al.*, 1994), RF1 (Ryden *et al.*, 1986), RF2 (Kawakami *et al.*, 1988) and RRF (this paper), or with conditional synthesis of factors such as IF1 (Cummings and Hershey, 1994), IF2 (Cole *et al.*, 1987), RRF (Janosi *et al.*, 1994) and IF3 (Olsson *et al.*, 1996). RRF inactivation and faulty ribosomal recycling may cause bacteriostasis by many possible mechanisms. Since RRF inactivation causes random re-initiation short distances downstream from a termination codon, inter-cistronic and post-cistronic regions of poly-cistronic mRNA will be translated. These new proteins may be harmful to the cell. Unplanned post-cistronic translation may also be harmful in other ways. On poly-cistronic messages, the re-initiating ribosomes may interfere with translation of downstream cistrons. Alternatively, ribosomes translating post-cistronically will eventually reach the 3′ end of an mRNA. This can occur under normal conditions when damaged mRNA (truncated within the cistron) is inadvertently used for protein synthesis. For the latter case, the cell has evolved a mechanism to eliminate the truncated protein and rescue the ribosome through the use of 10 Sa RNA which functions as tRNA as well as mRNA (Keiler *et al.*, 1996). In the absence of RRF however, this machinery cannot function to rescue ribosomes since ribosomes will simply re-initiate downstream from the 10 Sa termination codon and eventually reach the 3′ physical end. Ribosomes may then be pushed off from either mRNA or 10 Sa RNA in a form difficult for them to engage in the next round of translation.

Unlike the bacteriostasis observed when other protein synthesis factors are inactivated, RRF inactivation is bactericidal during the transition state from the stationary to the growing phase (Figure 2). As discussed above, *in vivo* inactivation of any one of the protein synthesis factors in *E.coli* does not cause a bactericidal effect. Likewise, most protein synthesis inhibitors, such as tetracycline and erythromycin, stop bacterial growth but do not kill bacteria. An exception reported is the bactericidal effect of *in vivo* inactivation of EF-G in *Bacillus subtilis* (Aharonowitz and Ron, 1972). It is tempting to speculate that this mutation in the *B.subtilis* EF-G gene is such that it specifically interferes with the ribosome recycling step in which EF-G participates (Hirashima and Kaji, 1973). Though the exact mechanism remains obscure, the bactericidal effect of RRF inactivation at the stationary phase is reminiscent of a similar effect of loss of ribosome modulation factor (RMF). Wada *et al.* showed that ribosomes at the stationary phase are maintained as 100S dimers by RMF (Wada *et al.*, 1995). If RMF is absent, stationary phase cells die but growing cells do not (Yamagishi *et al.*, 1993). It is possible that the lethal effect of inactivation of RRF and RMF at the stationary phase may be related.

The *in vivo* results detailed in this communication, in conjunction with previously published *in vitro* results, establish ribosomal recycling as the essential fourth step in prokaryotic protein synthesis. No direct information is currently available regarding an analogous step in eukaryotes; however, there are significant pieces of indirect evidence suggesting that prokaryotes and eukaryotes may complete the fourth step of protein synthesis in a different manner (discussed in Janosi *et al.*, 1996a). First, unlike prokaryotes, eukaryotic ribosomal subunits are believed to remain on mRNA when ribosomes pass through ORFs upstream from a cistron (Kozak, 1987). Unlike prokaryotes, initial binding of mRNA by ribosomes in eukaryotes is mostly limited to the 5′ cap region, and this region is frequently followed by an ORF before the cistron (for example, Lindahl and Hinnebusch, 1992). This makes it difficult to translate cistrons if ribosomes are released at the end of each ORF as observed in prokaryotes (Hirashima and Kaji, 1972a,b; Ogawa and Kaji, 1975).

Second, though homologs of *frr* exists in eukaryotic genomes (Janosi *et al.*, 1996b), eukaryotic RRF is found in only organelles such as mitochondria and chloroplasts (to be published elsewhere) which are believed to have originated from symbiotic bacteria during phylogenetic development. The notion that these eukaryotic RRF do not participate in cytoplasmic protein synthesis in eukaryotes is supported by a recent finding that *Methanococcus jannaschii* does not carry the *frr* equivalent (Bult *et al.*, 1996). This is the only reported bacterium which has eukaryotic protein synthesis machinery and carries neither the *frr* gene equivalent nor mitochondria, suggesting that the eukaryotic *frr* gene product does not participate in the cytoplasmic protein synthesis.

Given the likely difference between eukaryotic and prokaryotic ribosomal recycling described above, the fourth step of protein synthesis is an enticing new target for antimicrobial agents. RRF is especially suited as a target in view of the present observation that its inhibition could lead to bacterial death. Unlike other protein synthesis inhibitors such as tetracycline and erythromycin, the one which targets RRF and the fourth step of protein synthesis may be bactericidal. It should be noted that each of the first three steps of protein synthesis, namely initiation, elongation and termination, is sensitive to one or more antibiotics. One antibiotic, fusidic acid, is already known to inhibit the fourth step of protein synthesis *in vitro* because of its effect on EF-G (Hirashima and Kaji, 1973). As a first step towards developing antimicrobial agents directed at RRF, we are pursuing the three-dimensional structure of RRF. Preliminary nuclear magnetic resonance spectra data from large quantities of purified RRF (Hirashima and Kaji, 1972b; Ichikawa and Kaji, 1989) suggest that the three-dimensional structure is attainable by this approach. Preliminary attempts to obtain crystals of RRF have been successful. Once this novel protein's structure becomes available, antimicrobial drugs targeting it may be designed in a rational manner.

Materials and methods

Bacterial growth

Bacterial strains were grown as described (Janosi *et al.*, 1994). Media were supplemented with antibiotics such as tetracycline (TC, 12.5 μg/ml), chloramphenicol (CM, 20 μg/ml), kanamycin (KM, 50 μg/ml), ampicillin (AP, 100 μg/ml), 6% sucrose, 0.4 mM isopropyl-β-D-thiogalac-

toside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) when needed.

Bacterial strains and plasmids

Strains and plasmids were constructed with standard methods (Miller, 1972; Sambrook *et al.*, 1989). The *E.coli* strains and plasmids used in this study are shown in Table I. Plasmid pPEN728 contains the wild-type *frr* (gene coding for RRF) from pRR2 (Shimizu and Kaji, 1991). For pPEN874, the temperature-resistant replicon of pSC101 was inserted in place of the Ts replicon of pMAK705. Plasmid pPEN907 was constructed from pPEN874 by inserting the wild-type *frr* with its promoter (Shimizu and Kaji, 1991) and transcriptional termination signal (Ichikawa and Kaji, 1989). Plasmid pPEN1560 was constructed from pPEN728 by adding the *sacB*-Neo^r (sucrose sensitivity and KM resistance) cassette from pIB209 (Blomfield *et al.*, 1991) and deleting the 117 bp *Sca*I–*Xmn*I fragment inactivating the AP^r gene.

Isolation of temperature-sensitive frr alleles

The PCR reaction with error-prone *Taq* polymerase was carried out on pPEN907 with the M13/pUC19 universal sequencing primers. The product was digested with *Kpn*I and *Hin*dIII, ligated to pPEN874 resulting in a plasmid mixture carrying mutated *frr* and CM^r, (pMIX). *E.coli* LJ3 cells (*frr1* Fs, Fs stands for frame shift) harboring pPEN1560 (contains wild-type *frr*) were transformed with pMIX followed by selection for CM^r and sucrose-resistant transformants at 32°C and screened for temperature-sensitive growth at 43°C by replica plating. The chromosomal *frr* of MC1061 was replaced with Ts *frr* alleles on cloned plasmid as described earlier (Hamilton *et al.*, 1989; Janosi *et al.*, 1994) to obtain LJ13, LJ14, LJ15 and LJ16.

Semiquantitative characterization of the temperature-sensitive growth phenotype

E.coli strain LJ3 (*frr1*Fs) carrying the Ts *frr* alleles in pMW119 or LJ13, LJ14, LJ15 and LJ16 harboring Ts *frr* allele in the bacterial chromosome were grown overnight in Luria–Bertani broth (LB) medium (Janosi *et al.*, 1994) at 32°C. The cultures were diluted serially 10-fold and 15 μl aliquots were spotted onto Luria agar (LA; Janosi *et al.*, 1994) plates, incubated overnight at 32°C, 37°C and 43°C and the dilutions giving rise to 10–20 colonies were recorded.

RRF and other assays

Wild-type RRF from *E.coli* DH5α harboring pRR1 (carrying wild-type *frr*; Ichikawa and Kaji, 1989) and Ts mutant RRF from *E.coli* LJ14 harboring pPEN2399 (carrying Ts*frr*) were purified and assayed as described (Hirashima and Kaji, 1972b). Purified EF-G was a gift from Dr Barend Kraal (Faculty of Mathematics and Natural Sciences, Leiden Institute of Chemistry, Leiden, The Netherlands). Protein concentrations and β-galactosidase activity were measured by the DC Protein Assay kit (Bio Rad) and the Miller method (Miller, 1972), respectively.

lacZ′-′lacZ gene fusion and re-initiated products

Plasmids pPEN2345, pPEN2367, pPEN2363 and pPEN2369 were constructed from pLKC481 and pLKC480 (American Type Culture Collection) and pUC19 (Yanisch-Perron *et al.*, 1985). They have the following junction sequence: pPEN2345, P*lac* (promoter for *lac*)-RBS (ribosome binding sequence) -ATG-36 nucleotides-ACT-39 nucleotides-GTC (10th codon of *lacZ*)---; pPEN2367, P*lac*-RBS-ATG-36 nucleotides-ACT-37 nucleotides-GTC (10th codon of *lacZ*)---; pPEN2363, P*lac*-RBS-ATG-36 nucleotides-TAG-39 nucleotides-GTC (10th codon of *lacZ*)---; pPEN2369, P*lac*-RBS-ATG-36 nucleotides-TAG-37 nucleotides-GTC (10th codon of *lac Z*)---. The initiation codon ATG of pPEN2345 is in-frame with β-galactosidase (′*lacZ*) while that of pPEN2367 is in the –2 frame. The termination codon is at the 14th triplet of these plasmids (pPEN2363 and pPEN2369) and is in-frame with the initiation codon ATG. The termination codon TAG of pPEN2363 is in-frame with the downstream β-galactosidase (′*lacZ*) while that of pPEN2369 is in the –2 frame. The Edman analysis (six cycles) (Matsudaira, 1993) was carried out on 50–200 pmol of affinity-purified (Protosorb *lacZ*, Promega) β-galactosidase induced upon inactivation of RRF.

The 3A′ reporter system

The 3A′ plasmids pSM–T272 and pSM–T275 were constructed from plasmid pSM11 (Mottagui-Tabar *et al.*, 1994), pAB7 (Björnsson and Isaksson, 1993) and necessary synthetic nucleotides. They have the following junction sequence between the upstream 2A′ and the downstream 3A′ cistrons; pSM–T272; 2A′-**TAA**-8 nucleotides-GGT-9 nucleotides-**ATG**-3A′. pSM–T275; 2A′-**TAA**-9 nucleotides-GGT-9 nucleotides-**ATG**-3A′. Bold TAA is the termination codon for the upstream 2A′ segment and bold ATG is the initiation codon for the downstream 3A′ cistron. Procedures for growth of bacterial cultures harboring either pSM–T272 or pSM–T275, purification of protein-A′ species, separation on SDS–PAGE and determination of band densities were as described earlier (Björnsson *et al.*, 1996).

Plasmid pSEK336C derived from IS3

Plasmid pSEK336C was prepared from pSEK336 (Sekine *et al.*, 1994) by deletion of a *Hin*dIII fragment within the *cI857* gene to inactivate the repressor. Plasmid pSEK336C carries an IS*3* (insertion sequence 3) segment where the 3′ portion of *orfA* (′*orfA*) and the 5′ portion of *orfB (orfB′)* are allocated in a tandem array. The *lacZ* reporter gene is in-frame with *orfB*′ which is in –1 frame with respect to ′*orfA*. The IS*3* segment has the following sequence: **ATG**-16 nucleotides-AA**C**AG-29 nucleotides-**C**TGAA---. **C** represents the mutation changing the initiation codon ATG of *orfB*′ to CTG. The bold ATG and TGA are the initiation codon and the termination codon for ′*orfA* respectively. To avoid confusion with ribosomal frameshifting which occurs from *orfA* to *orfB* in the natural IS*3* sequence, the sequence causing the programmed frame shift has been eliminated by a mutation (the bold **C** above) (Sekine *et al.*, 1994). The cells harboring pSEK336C were grown at 39°C and the cultures were kept in the logarithmic growth phase (OD_{600} between 0.03 and 0.6). Purification of the induced β-galactosidase and amino-terminal sequencing was performed as described (Sekine *et al.*, 1994).

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