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Mutations Influencing the *frr* Gene Coding for Ribosome Recycling Factor (RRF)

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A total of 52 null, six reversion, and five silent mutations of *frr* (the gene encoding for ribosome recycling factor (RRF)) of *Escherichia coli* are discussed along with 12 temperature-sensitive (ts) mutations and 14 intergenic suppressor strains of ts RRF. The null mutations were classified into six different categories. A computer-based secondary structure analysis showed three domains; domain A which has the N-terminal helix, domain B which contains coil, α-helix and β-strand structure, and domain C which is a C-terminal helix. The ts mutations fell into domains A and C but not in domain B. More than a half of the null mutations fell into domain B while the silent mutations fell outside domain B. Substitution of Arg132 in domain C by other amino acids was observed among five independently isolated null mutants. It is suggested that domain B is important for maintaining the RRF structure, while the region including Arg132 is one of the active sites. A total of 14 intergenic suppressor strains of ts RRF were grouped into four categories, depending on which temperature-sensitive alleles were suppressed.

Keywords: frr; mutants; recycling; ribosome; RRF

Introduction

Ribosome recycling factor (RRF), elongation factor G (EF-G) and GTP disassembles the post-termination complex which consists of the 70 S ribosome bound to the termination codon of the mRNA and deacylated tRNA. This step may be described as the fourth step of protein synthesis because without this step the next round of protein synthesis does not occur (for reviews, see Janosi *et al.*, 1996a,b; Kaji *et al.*, 1998; Kaji & Hirokawa, 1999). Absence of RRF *in vitro* (Ryoji *et al.*, 1981) as well as *in vivo* (Janosi *et al.*, 1998) leads to the unscheduled reinitiation of translation of mRNA downstream from the termination codon. Furthermore, absence of RRF in the *in vitro* translation system caused increased translational errors (Janosi *et al.*, 1996b). Recent evidence suggests that release factor 3 (RF3) can replace EF-G in the reaction catalyzed by RRF (Grentzmann *et al.*, 1998). RRF is essential for bacterial growth (Janosi *et al.*, 1994); its absence leads to cell death under certain conditions (Janosi *et al.*, 1998). Its homologue is present in all eubacteria thus far examined (Himmelreich *et al.*, 1996; Kaneko *et al.*, 1996; Vizcaino *et al.*, 1996; Blattner *et al.*, 1997; Fraser *et al.*, 1997; Kunst *et al.*, 1997; Deckert *et al.*, 1998; Lowe *et al.*, 1998; Ohnishi *et al.*, 1999; for a review, see Janosi *et al.*, 1996) but is absent from Archea (Bult *et al.*, 1996; Klenk *et al.*, 1997) which have eukaryotic protein biosynthesis apparatus. In eukaryotes, the RRF homologue is confined to such organelles as mitochondria (Kanai *et al.*, 1998) or chloroplasts (Rolland *et al.*, 1999) but is not essential under certain conditions (Teyssier *et al.*, 1998). These attributes make RRF a potential target for anti-bacterial agents.

Although the biochemical reaction mediated by RRF has been characterized in our work (Hirashima & Kaji, 1972, 1973; Ogawa & Kaji, 1975; Janosi *et al.*, 1994; Ryoji *et al.*, 1981; Ohnishi *et al.*, 1999) as well as in classical and recent contributions from other laboratories (Kung *et al.*, 1977; Pavlov *et al.*, 1997; Heurgué-Hamard *et al.*, 1998; Karimi *et al.*, 1999), little information is available on the structure function relationship of this

Abbreviations used: RRF, ribosome recycling factor; ts, temperature-sensitive; EF-G, elongation factor G; RF3, release factor 3; CM, chloramphenicol; KM, kanamycin; AP, ampicillin; tr-large, temperature resistant large; tr-small, temperature resistant small; ts, temperature sensitive allele; wt, wild-type.

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molecule. Several examples in the literature show that understanding the function and structural organization of a protein and its interacting proteins (Jonczyk *et al*., 1998) is greatly facilitated by characterizing the mutationally altered proteins. For this reason, we have previously isolated RRF mutants that show temperature-sensitive growth phenotypes (Janosi *et al*., 1998). Here, we report additional mutant classes of RRF and discuss the possible implications for the role of these mutated amino acids in the structure, function and the possible interactions of RRF with other cellular components.

Results

Temperature-sensitive *frr* mutations were not located in domain B of RRF containing the β-strand predicted by computer

The computer-predicted (Geourjon & Deleage, 1995) secondary structure of RRF (Figure 1) shows that the molecule has at least three structural domains. The N-terminal α-helical domain A (amino acid positions 1 to 27) is followed by domain B in which short stretches of coiled, β-strand and α-helical structure alternate (amino acid positions 28 to 106). The third is a huge α-helical domain C, (amino acid positions 107 to the end) which may be disrupted by a short coil structure (amino acid positions 147 to 149, Figure 1). All of the temperature-sensitive *frr* (ts*frr*) alleles characterized previously (Janosi *et al*., 1998) are localized in either domain A (alleles 8 and 15) or C (alleles 14, 2, 6, 7, 4, 10, 16, 17 and 13), or in both of these domains (allele 3). This suggests the possibility that domain B and the ends of domain A and C (the region, positions 22 to 116 in Figure 1) are such that even a slight change of amino acids may be fatal, thus making it impossible to have temperature-sensitive mutations in this region.

Isolation of null mutants of frr

To isolate plasmids carrying null mutations in *frr*, we started with plasmid pMIX which carries the chloramphenicol (CM) resistance marker and mixtures of mutated *frr* as described previously (Janosi *et al*., 1998). The *E. coli* strain used was LJ4 (*recA*$^-$) with a frame-shifted chromosomal *frr*. This strain harbors pPEN1560 which carries wild-type *frr* (wt*frr*), the kanamycin (KM) resistance marker and the sucrose sensitivity marker. These bacteria were transformed with pMIX, and the transformants were selected on LA plates with CM at 32 °C. Since *frr* is essential for bacterial growth (Janosi *et al*., 1994) and LJ4 has a non-functional chromosomal *frr*, we reasoned that LJ4 harboring pMIX which carries null mutations of *frr* would require pPEN1560 which carries functional *frr*. We, therefore, looked for cells which retain both plasmids, pMIX and pPEN1560, despite the fact that these plasmids are incompatible with each other.

The transformant colonies obtained above were replica plated onto LA containing sucrose supplemented with CM and on LA plates with CM and KM. Colonies growing on KM and CM plates but not on CM and sucrose were passed through three consecutive passages on LA plates with CM only, and were scored for the unselected KM resistance marker. Altogether 153 colonies were found which retained the unselected KM resistance marker (the marker of the resident pPEN1560 plasmid encoding wild-type RRF). These colonies were considered to harbor a pMIX plasmid carrying non-functional *frr* and the CM resistance gene. Hence, they are forced to maintain pPEN1560 which carries a functional *frr* despite the fact that pMIX and pPEN1560 are incompatible with each other.

Each plasmid DNA (a mixture of pMIX and pPEN1560) from 153 transformants was purified and used to transform the *E. coli* DH5α strain. Plasmid pPEN1560 is not needed by *E. coli* DH5α because the strain carries the wild-type *frr* on the chromosome. Therefore, transformants carrying only the pMIX plasmid were mostly expected upon screening by CM. To screen for KM sensitive colonies (therefore without pPEN1560) on this plate, they were replica plated on plates containing KM. From each of 153 KM sensitive strains (which harbors only pMIX carrying null *frr*) thus isolated, pMIX plasmids were purified. The plasmids were digested with *Kpn*I and *Hin*dIII, and 61 isolates from transformants in which pMIX carried the 0.9 kb *Kpn*I-*Hin*dIII null *frr* fragment were sequenced. As can be seen in Table 1, these 61 isolates showing alteration from the wild-type sequence represented 52 different genotypes.

The isolated mutants were confirmed to be null mutants because none of them were shown to complement LJ14 at 42 °C. The vector in which null mutated *frr* is placed was shown to be functional because the wild-type *frr* placed in the same vector expressed the RRF function. We therefore concluded that these mutations in *frr* must be null mutations. It is possible that the inactivity of these null *frr*s are due to cellular proteolytic digestion of the unnatural gene product which may have been rendered susceptible to proteolysis; alternatively, the mutated gene products are indeed inactive. In discussing the functional inactivity of null *frr* in this communication these two possibilities are not distinguished.

C-terminal truncations

Out of the 61 isolates, 37 had C-terminal truncations in various lengths. These 37 isolates had 34 different genotypes as shown in Table 1. The longest molecule with a C-terminal truncation and without any further alteration in the primary structure of RRF was coded for by the *frr142* allele (class D in Table 1). This truncated molecule has 171 amino acid residues indicating that the intact N-terminal 171 amino acid residues of the RRF molecule is not enough for the RRF function. All

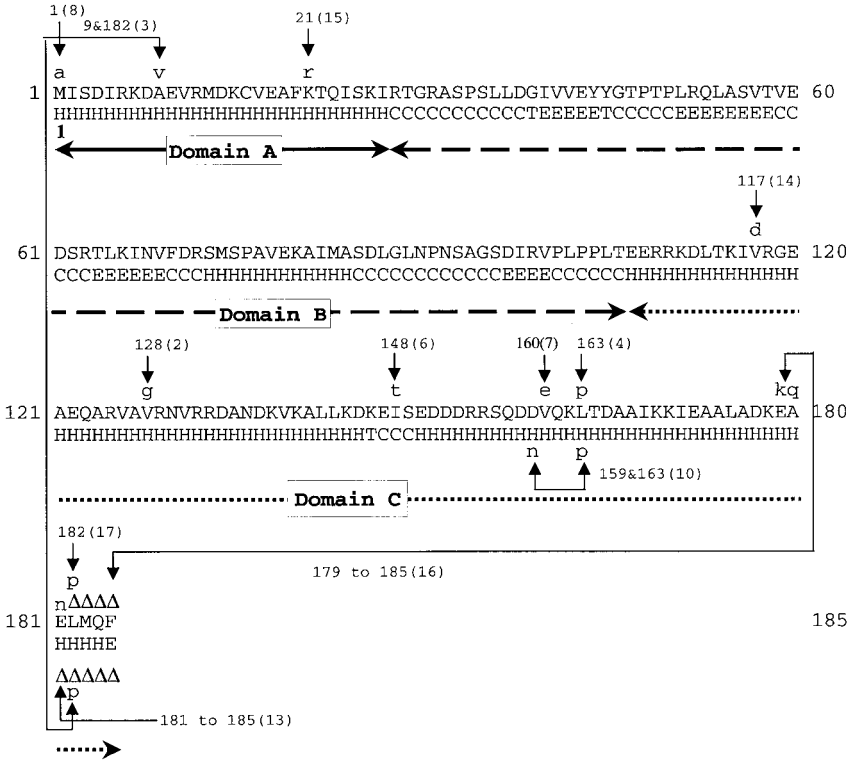

Figure 1. Relationship of the positions of various mutations with the primary sequence (Ichikawa & Kaji, 1989) and a computer-predicted secondary structure of RRF with the SOPMA method (Geourjon & Deleage, 1995). Mutations, conferring temperature-sensitive cell growth, were identified earlier (Janosi *et al.*, 1998). The amino acid residues of the mutant *frr* alleles are indicated with one letter code on top of the wild-type amino acid position. Arrows indicate the position of the amino acid residue alteration. The first number at the end of the arrow indicates the residue number. The number in the parentheses is the allele number (Janosi *et al.*, 1998). The open triangle indicates the absence of the particular amino acid residue from the sequence of the mutant. If more than one change occurs in the sequence of the allele, the arrows are connected with continuous lines. H, α-helix; C, coil; E, strand; T, turn.

alleles which code for a C-terminal truncation with additional single amino acid changes (class E) code for shorter RRF molecules than the *frr142* allele. This suggests that the additional single amino acid alterations are not able to compensate for the functional deficiency due to long C-terminal truncation.

The *frr135* allele (in class F) encodes a molecule 181 amino acid residues long but the C-terminal 14 amino acid residues are changed because of frameshift mutation at the 511 nucleotide position. This molecule is longer than the one encoded for by *frr142* and *frr13* (temperature-sensitive RRF with 180 amino acid residues and no other mutational change, see Figure 1 for the amino acid sequence and ts *frr13*). Despite the length of the molecule, the preserved wild-type sequence in *frr135* (167 residues) is just too short for the RRF function while ts *frr16* (178 residues) is functional at the permissive temperature.

Single amino acid alterations and hot spots of mutations

As shown in Table 1 as class A, there were 11 alleles with single amino acid change mutations which were found most frequently among all the null mutations. The arginine residue at position 132 is a hot spot for this type of mutation because it appeared four times (*frr114, frr132,* twice *frr133*). It also occurred in one of the mutants with a triple amino acid change (*frr141*). Preservation of an arginine residue at this position is important because substitution of an arginine residue by other amino acid residues such as Cys, Gly or His lead to null mutations. The null R132H mutation shows that preserving positively charged amino acids at this position is not sufficient to keep the RRF activity.

Other multiple occurrences of a single amino acid substitution, though not as hot a spot as Arg132, were at positions 65 and 175; Leu65Pro (*frr161* and *frr109*) and Leu175Pro (*frr124* and *frr149*). Mutations responsible for *frr146, frr160, frr106* and *frr119* alleles were observed only once.

Double and triple amino acid alterations

There were six isolates with double amino acid substitutions, appearing in five different genotypes (class B in Table 1). In one of these (*frr118*), Arg110His substitution occurs in addition to the Asn90Ser substitution. Because the Arg110His substitution alone is a null mutation (*frr106* allele (see class A)), this means that the Asn90Ser substitution did not convert this mutated protein into an active one. A similar situation exists with triple mutations. One of the two mutants with three amino acid changes (class C in Table 1) carries the Arg132Cys substitution (*frr141*) which alone is a null mutation (*frr132* in class A). Hence, the additional changes Gly30Ser and Ser33Pro did not rescue the null mutation at Arg132Cys. Other alleles involved in double or triple mutations did not involve any of the single amino acid change null mutations, suggesting that it is the concerted

Table 1. Genotypes of non-functional frr mutations

Mutation (Class)	Allele	N[a]	Nucleotide change	Change in the primary sequence of RRF	Notes
Single AA change	*frr146*	1	T152C	Leu51Pro	-
	frr160	1	T161C	Leu54Pro	-
	frr161	1	T194C	Leu65Pro	-
	frr109	1	T99C T194C	Leu65Pro	Ser33 silent
	frr106	1	C123T G329A	Arg110His	Val41 silent
	frr119	3	C−91A[b] C385T	Arg129Cys	Promoter involved
(Class A)	*frr114*	1	C394G	Arg132Gly	-
	frr132	1	C394T	Arg132Cys	-
	frr133	2	G395A	Arg132His	-
	frr124	2	T524C	Leu175Pro	-
	frr149	2	C447T T524C	Leu175Pro	Ser149 silent
Double AA change	*frr165*	1	G28A A490C	Glu10Lys Thr164Pro	-
	frr113	1	G28C G162A A490C	Glu10Lys Thr164Pro	Leu154 silent
(Class B)	*frr112*	1	T38C T512C	Met13Thr Ile171Thr	-
	frr116	2	T107A C317A	Leu36Gln Thr106Lys	-
	frr118	1	A269G G329A	Asn90Ser Arg110His	-
Triple AA change	*frr134*	1	T14C A103G T194A	Ile5Thr Ser35Gly Leu65Glu	-
(Class C)	*frr141*	1	G88A T97C C394T	Gly30Ser Ser33Pro Arg132Cys	-
C-terminal truncation *via*	*frr127*	1	G52T T416C	Glu18stop (17 AA long RRF)	+mutation beyond stop
early stop	*frr123*	1	A76T	Lys26 stop (25 AA long RRF)	-
	frr158	1	C135G	Tyr45 stop (44 AA long RRF)	-
	frr125	1	C157T	Gln53 stop (52 AA long RRF)	-
	frr162	1	T24C C157T	Gln53 stop (52 AA long RRF)	Asp8 silent
(Class D)	*frr140*	1	A196T T206C G309T	Lys66 stop (65 AA long RRF)	+2 mutations beyond stop
	frr110	1	C218A	Ser73 stop (72 AA long RRF)	-
	frr131	1	C−6A C218A	Ser73 stop (72 AA long RRF)	SD-initiation spacer involved
	frr108	1	G364T A540G	Glu122 stop (121 AA long RRF)	-
	frr117	1	A430T	Lys144 stop (143 AA long RRF)	-
	frr136	1	T336C A430T	Lys144 stop (143 AA long RRF)	Asp112 silent
	frr159	1	T−46C A508T	Lys170 stop (169 AA long RRF)	+mutation between promoter and SD
	frr142	3	G514T	Glu172 stop (171 AA long RRF)	-
C-terminal truncation &	*frr115*	2	A103G C157T	Ser35Gly Glu53 stop (52 AA long RRF)	-
single AA change	*frr151*	1	A11G G364T	Asp4Gly Glu122 stop (121 AA long RRF)	-
	frr140	1	A61G C367T	Lys21Glu Glu123 stop (122 AA long RRF)	-
	frr166	1	A61G G162A C367T	Lys21Glu Gln123 stop (122 AA long RRF)	Leu154 silent
(Class E)	*frr121*	1	A445G C469T	Ser149Gly Gln157 stop (156 AA long RRF)	-
	frr152	1	C467T C469T	Ser156Phe Gln157 stop (156 AA long RRF)	-
	frr139	1	C467T C469T C555T	Ser156Phe Gln157 stop (156 AA long RRF)	mutation beyond stop

Table 1. (*continued*)

C-terminal truncation preceded by frame-shift	*frr170*	1	T5 del Stop at nt 50-52	(16 AA long RRF)	Ser3 to Cys16 changed
	frr101	1	G40 del Stop at nt 50-52	(16 AA long RRF)	Asp14 to Cys16 changed
	frr169	1	A70 del Stop at nt 170-172	(56 AA long RRF)	Ile24 to Ser56 changed
	frr143	1	A79 del Stop at nt 170-172	(56 AA long RRF)	Ile27 to Ser56 changed
	frr129	1	C75del Stop at nt 170-172	(56 AA long RRF)	Ser25 to Ser56 changed
	frr126	1	C101 del A419T Stop at nt 170-172	(56 AA long RRF)	Ser35 to Ser56 changed +mutation beyond stop
(Class F)	*frr105*	1	T170 del A359T T492C Stop at nt 176-178	(58 AA long RRF)	Val57 to Thr58 changed +2 mutations beyond stop
	frr147	1	CG82-83 del Stop at nt 210-212	(69 AA long RRF)	Arg28 to Val69 changed
	frr153	1	AT199-200C Stop at nt 266-268	(88 AA long RRF)	Ile67 to Gly88 changed
	frr107	1	A333 del Stop at nt 338-340	(112 AA long RRF)	Asp112 changed
	frr103	1	C362 del Stop at nt 416-418	(138 AA long RRF)	Ala121 to Lys138 changed
	frr137	1	A346 del A363G A501G Stop at nt 416-418	(138 AA long RRF)	Ile116 to Lys138 changed +mutation in shifted sequence +mutation beyond stop
	frr145	1	A389 del Stop at nt 416-418	(138 AA long RRF)	Asn130 to Lys138 changed
	frr135	1	A511 del Stop at nt 545-547	(181 AA long RRF)	Ile168 to Glu181 changed

In description of nucleotide and amino acid changes, the positions of the changes are indicated by a number preceded by a description of the wild-type nucleotide or amino acid residue and followed by mutated ones. The amino acid residues are represented by their three letter codes. Abbreviations: AA, amino acid; del, deletion.

[a] N = number of isolates.

[b] This change may not influence the promoter activity because A in this position (sixth position in the −35 sequence of the promoter) is commonly A (Harley & Reynolds, 1987).

effects of a two or three amino acid changes which may lead to the loss of activity in these cases.

Critical and non-critical regions of the RRF molecule

As discussed above, null mutations are found in the region where the temperature-sensitive mutations are not observed (the shaded area as shown in Figure 2). This region is tentatively called a ''forbidden region'' because temperature-sensitive mutations are ''forbidden'' (not found) in this region. On the other hand, the fact that more than a half of the total mutated positions (12 out of 21, excluding the C-terminal truncation) are mapped in this region suggests the importance of this region for maintaining the RRF activity. The fact that not one of the mutations which do not influence the RRF activity (silent mutations) is found in this region also supports the importance of this region.

Another critical region for the activity is the region 129-132. As described in the section on single amino acid alterations, the position 132 is a hot spot. In addition, we have two more mutations in this narrow region. The Arg129Cys substitution (*frr119*) is null and the Val128Gly (ts *frr2*) alteration is a temperature-sensitive mutation. It is plausible therefore that the mutations in the Arg129-Arg132 region are all null mutations while changes nearby such as Val128Gly renders RRF unstable through the same mechanism but with a less severe effect.

As pointed out in the section on C-terminal truncations, the C-terminal end of the molecule is important for the RRF activity as indicated by the *frr142* allele (this lacks the C-terminal 14 amino acid residues). This notion is further strengthened by the existence of the Leu175Pro substitutions (in *frr124* and *frr149*) leading to null mutations. However, the extreme C-terminal end may not be essential because mutations further downstream from position 179 seem not to be vitally important because no null mutation maps there. Instead, we find several temperature-sensitive mutations in this region, suggesting the stabilizing role of this region against thermal insult.

In contrast, strict preservation of the N-terminal region is probably not important for the RRF activity because we did not find any lethal single amino acid substitutions in the N-terminal region. However, mutations in the N-terminal region rendered the RRF molecule sensitive to elevated temperatures (Met1Ala, Ala9Val and Lys21Arg). Met1Ala suggests that the RRF coded for by this allele (ts*frr8*) has a 12 amino acid truncation because methionine at the 13th position probably serves as an initiation codon. It is possible that the Ala9Val substitution alone may not have any effect because this change is accompanied by C-terminal truncation (three amino acids residues) in ts *frr3*. Although we find amino acid substitutions in the N-terminal region among null mutations, they are always accompanied by a second amino acid substitution in Domain C (as in the *frr112*, *frr165* and *frr113* alleles). Similarly, the amino acid substitution I5T is accompanied by two more substitutions in domain B (*frr134*). These mutations suggest again that changes at the N-terminal alone are not sufficient to render the molecule non-functional.

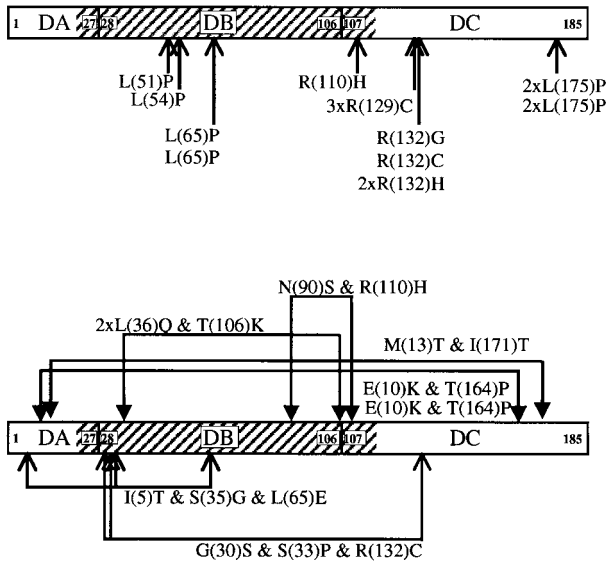

Figure 2. Distributions of null mutations in three computer predicted domains of RRF. Three structural domains, Domains A (DA), B (DB) and C (DC) are indicated by three boxes. The numbers within the domain boxes indicate the starting and the ending amino acid positions of the domains. The shaded area indicates the region, amino acid position 22 to 116 where temperature-sensitive mutations do not occur (forbidden). The null mutations with a single amino acid change are shown below the box of the upper panel. The lower panel shows the positions of the null mutations with double amino acid changes (above the box) and triple amino acid residue changes (below the box). The amino acid changes are indicated at the end of arrows by the notation with the wild-type amino acid residue written on the left of the parentheses (which gives the location of the change) and the changed amino acid residue written on the right.

Reversion of the temperature-sensitive phenotype to wild-type

Rate of reversion of the temperature-sensitive phenotype of E. coli *and isolation of two types of temperature resistant revertants*

For studies on reversion of temperature-sensitive *E. coli* with ts *frr14*, *E. coli* LJ4 harboring pKH6 was used. *E. coli* LJ4 has the null frame shift mutation in its chromosomal *frr* (*frr1*) and hence is dependent on a plasmid which carries functional *frr* (Janosi *et al.*, 1994). Plasmid pKH6 carries ts *frr* (*frr14*) in addition to the ampicillin (AP) resistance marker. *E. coli* LJ4 harboring pKH6 is, therefore,

temperature-sensitive. This strain was grown overnight at 27 °C and plated on LA plates, then incubated at 31 °C or at 43 °C for two nights. The number of colonies on the plates at 43 °C was 4.2×10^{-6} of that on the plates incubated at 31 °C. Since the copy number of pKH6 is five to six, the actual reversion rate of LJ14 with single ts *frr14* in the chromosome may be lower than this frequency.

The colony at 43 °C in the above experiment varied in size from pinpoint to normal (3-4 mm in diameter). At 31 °C, the size of the colonies was more uniform and was of the normal (3-4 mm in diameter) type. A total of 50 normal size (called temperature resistant large (tr-large)) and 50 small size (called temperature resistant small (tr-small)) colonies from the 43 °C culture were passed through three consecutive single colony passages at 43 °C. The colony characteristics remained unchanged during this passage, indicating that this phenotype is stable. We selected 14 of each kind and named them tr-large 1 through 14 and tr-small 1 through 14.

Sorting the temperature resistant revertants: tr-large strains are intragenic revertants

We predicted that those revertants with normal size colonies would mostly have intragenic reversion of Val117Asp to valine because this would be a simple restoration of the normal RRF activity. On the other hand, we expected that any genetic change of other cellular genes to compensate for the temperature-sensitive mutation of RRF may not be sufficient to restore the normal functions of *frr* at 43 °C. In addition, these changes may be deleterious to the normal functions of this gene at 43 °C. If so, one would expect a slower growth, hence a smaller colony. In addition, intragenic mutation at the positions other than residue 117 (ts *frr14* has Val117Asp) may restore the RRF activity partially, resulting in a small colony.

All of our revertants, as described above, had the frameshift mutation in the chromosomal *frr* and were dependent on the functional *frr* on the plasmid pKH6. We therefore reasoned that those revertants with intragenic reversion but not with intergenic suppression, would revert back to the temperature-sensitive phenotype if we replace the resident plasmid (pKH6) with a plasmid carrying ts *frr*. Therefore, tr-large strains 1 through 14 and tr-small strains 1 through 14 were transformed with the plasmid pPEN1586 (this carries the temperature-sensitive *frr14* (Ts) allele and the CM resistance marker). This plasmid is incompatible with the resident AP resistance plasmid pKH6. The transformants were passed through three consecutive single colony passages screening only for the incoming CM resistance plasmid to segregate the resident plasmid by the plasmid incompatibility principle. As shown in Table 2, all tr-large strains (line 1), but not tr-small strains (line 2), became temperature-sensitive. To verify that the resident pKH6 AP resistance plasmid had indeed been eliminated from them, the colonies were replica plated onto LA plates supplemented with AP. None grew on this plate (see Table 2, column 4).

The nucleotide sequences of *frr*s carried by pKH6 which gives rise to the large colony at 43 °C is shown in Table 3. Without exception, the nucleotide in position 350 changed back from adenine (A) of the *frr14* temperature-sensitive allele to thymine (T) of the wild-type. This restored the wild-type valine (GTT) in position 117 in place of aspartic acid (GAT) of ts *frr14*. This restoration was the only change in allele *frr201*.

As shown in Table 3, further amino acid changes observed in these revertants had no impact on the RRF function. Such changes were Asp153Tyr (in *frr206* and *frr204*), Asp153Ala (in *frr205*), Ile171Phe (in *frr206* and *frr205*) and Ile171Leu (in *frr204* and *frr202*). It is noted that the changes at positions 153 and 171 occurred in eight and 11 out of 14 isolates, respectively. These changes include radical changes in the character of the amino acid residues such as Asp153Tyr, suggesting that various amino acid structures at these positions are acceptable in maintaining the functional integrity of RRF. These mutations were called silent mutations because they did not manifest any phenotypes. It should be noted that these ''silent mutations'' were not selected but accidentally accompanied the

Table 2. Differential effect of pPEN1586 carrying *tsfrr14* on tr-large and tr-small strains: evidence that tr-large strains are intragenic revertants

			After the selective pressure for the transforming plasmid[a]	
Strain	Resident plasmid	Transforming plasmid	Presence of pKH6 (AP resistance)	Growth at 43 °C
Tr -large 1 through 14	pKH6	pPEN1586	−	−
Tr -small 1 through 14	pKH6	pPEN1586	−	+

[a] tr-large and tr-small 1 through 14 (see the text) harboring pKH6 which carries AP^r and ts*frr* (may be mutated) were transformed with pPEN1586 which carries CM^r and ts*frr14*. Eight transformants were selected and maintained for 70-80 cell generations (three consecutive passages of single colonies) under selective pressure for the transforming chloramphenicol resistance (CM^r) plasmid. Then, the presence of the ampicillin resistance (AP^r) resident plasmid (pKH6) at 32 °C and the growth capability of the colonies at 43 °C were probed by replica plating. The minus sign in the AP resistance column means the absence of pKH6. The plus and minus signs in the last column indicate growth and no growth, respectively.

Table 3. Genotypes of intragenic suppressor mutations reverting the temperature sensitive phenotype

			Nucleotide in position					Amino acid in position			
Allele	Phenotype	Number of isolates	350	457	458	511	513	117	153	171	Notes
frr	wt	NA	T	G	A	A	T	Val	Asp	Ile	Ichikawa & Kaji (1989)
frr14	ts	NA	A	G	A	A	T	Asp	Asp	Ile	Janosi *et al.* (1998)
frr201	tr revertant	2	T	G	A	A	T	Val	Asp	Ile	-
frr206	tr revertant	3	T	T	A	T	T	Val	Tyr	Phe	-
frr204	tr revertant	1	T	T	A	T	A	Val	Tyr	Leu	-
frr202	tr revertant	3	T	G	A	T	A	Val	Asp	Leu	-
frr203	tr revertant	1	T	G	A	A	A	Val	Asp	Ile	Ile171 silent
frr205	tr revertant	4	T	G	C	T	T	Val	Ala	Phe	-

Amino acids are represented by their 3-letter codes. Abbreviations: NA, not applicable; wt, wild-type; ts, temperature sensitive; tr, temperature resistant.

reversion at position 117 because all of our screening was designed for mutations with functional impact.

Evidence that tr-small strains carry intergenic suppressor mutations

As described above, when the pKH6 plasmids in tr-small strains were replaced with pPEN1586 (carrying ts *frr14)*, they were not converted to the ts phenotype (Table 2, line 2). We therefore concluded that the mutational change of tr-small strains resides outside of the resident plasmid pKH6. Since the revertants contain another *frr* allele (*frr1*) in the bacterial chromosome in addition to the one in the pKH6, mutational change(s) in *frr1* could cause the reversion. We reasoned that if the reversion is due to the functional restoration of the chromosomal *frr*, then these revertants would no longer need ts *frr*(*frr14*) carried by pKH6 at 43 °C or 32 °C.

The tr-small strains harboring pPEN1586 carrying *frr14* (ts) and CM resistance gene, used in the experiment described in Table 2, were transformed with plasmid pMW119 carrying the AP resistance gene but no *frr* related sequence (empty vector). In experiment A of Table 4, the transformants were cultured at 32 °C in the presence of AP (screens for the transforming pMW119). They were then replica plated on plates containing CM but no AP. All of them grew, indicating that all harbored pPEN1586 carrying the CM resistance gene. This shows that these two plasmids were maintained in the presence of AP alone without CM despite their incompatibility. Hence, these cells need the *frr14* gene carried by pPEN1586 for their growth.

In experiment B, the transformants obtained in A were passed through three consecutive single colony isolations on the plate containing CM but no AP at 32 °C. As shown in this Table, all of them grew showing that the resident plasmid pPEN1586 remained. All of them, however, lost pMW119 as shown by the fact that they were unable to grow on the AP plate. This established that the plasmid incompatibility principle works in this system and supports the conclusion reached from experiment A.

In experiment C, the transformants from experiment A were cultured as in experiment B but without any antibiotics, and the presence of each plasmid was examined by replica plating. Despite the absence of antibiotics, all of them retained plasmid pPEN1586 carrying ts*frr* and the CM resistance gene but segregated pMW119. We conclude that chromosomal *frr1* remained non-functional in tr-small strains and, therefore, these strains carry intergenic suppressor mutations. It should be noted that all transformants throughout experiments A, B and C grew at 43 °C, indicating that their temperature resistant character was maintained.

Classification of suppressor mutations

The data presented in Table 5 indicate that more than one suppressor gene is involved among 14 tr-small strains carrying ts *frr14*. Plasmid (pPEN1586, with ts *frr14*) harbored by all 14 tr-small strains were replaced with various plasmids (pKH1, 5, 8, 10 and 16 carrying ts *frr15*, *13*, *4*, *6* and *7*, respectively). Four independent isolates of newly created strains were tested for growth at 42 °C. The plus signs indicate growth of the strains at 42 °C.

It is noted in this Table that the 14 intergenic suppressor mutants isolated could be classified into four categories. Category A (tr-small 1, 3, 9, 11, 12, 13 and 14) represents the suppressor which suppresses all six *frr* temperature-sensitive mutations including *frr14*. Categories B (tr-small 2, 5 and 6) and C (tr-small 4) are the suppressors which suppress all but ts *frr15* or *frr7*, respectively. Lastly, category D (tr-small 7) represents the suppressor which could not suppress ts *frr4* and *frr15*. Growth of tr-small 8 and 10 harboring pKH1 (carrying *frr15*) was variable but grew at 43 °C with all other temperature-sensitive alleles. Therefore, these strains are categorized as belonging to either A or B.

Discussion

Relationship between *frr* mutations with the computer predicted secondary structure and biological activity of RRF

To evaluate the relationship between various mutations in RRF and the computer-predicted secondary structure of RRF, the secondary structure was constructed with the SOPMA method (Geourjon & Deleage, 1995) as shown in Figure 1. There are 19 regions representing helix, coil, turn and strand. Other prediction methods (Garnier, 1978; Deleage & Roux, 1987; Gibrat *et al*., 1987; Levin & Garnier, 1988; Geourjon & Deleage, 1994; Frishman & Argos, 1996) basically support this structure.

Although the exact effect of each mutation on the secondary structure is not reliable and therefore not shown, it was found that the lethal mutations changed the computer predicted secondary structure of domain B more than the other two domains A and C combined. Since domain B has only 78 amino acid residues out of a total of 185 amino acid residues of RRF, this observation suggests that the lethal mutations may cause more structural changes in domain B than domain A. This suggests that the maintenance of the domain B structure containing the mixture of coil, strand, short helix and turns may be important in maintaining the functional molecular integrity of RRF. Lethal mutations L51P, L54P and L65P (double) are all in the well-preserved leucine valine residues in

Table 4. Tr-small strains carry intergenic suppressor

Strain	Resident plasmid (CM^r)	Transforming plasmid (AP^r)	Presence of plasmids and growth capability at 43°C after maintaining selection for[a] (A) Transforming plasmid			(B) Resident plasmid			(C) None		
			pMW119	pPEN1586	Growth at 43 °C	pMW119	pPEN1586	Growth at 43 °C	pMW119	pPEN1586	Growth at 43 °C
Ts-small 1 through 14	pPEN1586	pMW119	+	+	+	−	+	+	−	+	+

[a] tr-small 1 through 14 harboring pPEN1586 (CM^r) were transformed with pMW119 carrying AP^r, four transformants were isolated and maintained for approximately 70-80 cell generations (three consecutive passages of single colonies) under selective pressure for the transforming ampicillin resistance (AP^r). Then the presence of the resident chloramphenicol resistance (CM^r) plasmid and the growth capability at 43 °C were probed by replica plating (experiments A). From this point, the colonies were maintained for another 70-80 cell generations under selective pressure for the resident CM^r (experiments B) plasmid or under no selective pressure for either of the plasmids (experiments C). Growth capability at 43 °C and the presence of plasmids were examined. The plus signs under plasmids indicate the presence of that plasmid.

Table 5. Intergenic suppressor of *frr* 14 may not suppress other temperature *frr* alleles

Host cell	Transforming plasmid (allele)					Type
	pKH1 (*frr*15)	pKH5 (*frr*13)	pKH8(*frr*4)	pKH10 (*frr*6)	pKH16 (*frr*7)	
	Growth at 42°C					
Tr-small 1	++++	++++	+++	++++	++++	A
Tr-small 2	−−−−	++++	++++	++++	±±++	B
Tr-small 3	++++	+++	++++	++++	++++	A
Tr-small 4	++++	++++	++++	++++	±−−−	C
Tr-small 5	−−−−	++++	+++±	++++	++±±	B
Tr-small 6	−−−	++++	+++±	+++	++±±	B
Tr-small 7	−−−−	+±±±	−−−−	±± ±	++±±	D
Tr-small 8	−−++	++++	++++	++++	++++	A or B
Tr-small 9	++++	+++	++++	++++	++++	A
Tr-small 10	−++−	+++	++++	++++	++++	A or B
Tr-small 11	++++	+++	++++	++++	++++	A
Tr-small 12	++++	++++	+++	++++	++++	A
Tr-small 13	++++	++++	+++±	++++	++++	A
Tr-small 14	++++	++++	++++	++++	+ ++	A

Strains were derived as described in Materials and Method. +, growth; −, no growth; ±, the growth was slow. Each entry represents four or three plates and the growth of each plate are indicated by the signs.

domain B. Changing these residues to proline residues are expected to cause a drastic change in the three-dimensional structure of this domain. It is therefore tempting to speculate that the maintenance of the structure involving these residues is crucial for the active RRF structure.

On the other hand, domains A and C consist mostly of three helices which probably contribute towards the heat stability of the molecule. In fact, RRF has been found to be a heat stable protein (Hirashima & Kaji, 1972). This hypothesis explains why temperature-sensitive mutations are localized in these domains. In further support of this notion, the mutation Val117Asp, converted the RRF homologue of *Staphylococcus aureus* to a temperature-sensitive protein (Lowe *et al*., unpublished results). In addition to contributing to heat stability, these domains, especially domain C, which contains Arg132, may be important as the active site of this molecule for interaction with other biological components such as ribosomes. Therefore, mutations in these regions by themselves may suffice to make the mutated RRF non-functional without causing much structural change. For example, the hot spot mutation *frr133* (Arg132His), does not cause any alteration in the computer predicted RRF secondary structure throughout the entire molecule. We propose that Arg132 may be an active site of RRF. Mutations near this position (positions 128 (ts *frr*2) and 129 (*frr119*)) produced either temperature-sensitive or null mutations. Every prokaryotic RRF thus far examined to date has Arg at the position 132 (for examples, see references given in the Introduction), indicating strong conservation of this residue during phylogenetic development. Furthermore, computer analysis indicated that Arg132 was exposed on the surface of the protein because it is situated in one of the hydrophilic regions (Kaji & Hirokawa, 1999).

Intragenic reversion of ts *frr14*

All 14 intragenic revertants had a single nucleotide change (A350T) that results in Asp117Val by changing the triplet sequence from mutated sequence GAT to wild-type sequence GTT. Intragenic revertants with other amino acid changes leaving Asp117Val intact were not found either among tr-small or tr-large strains. This can be explained by the fact that the Asp to Val change requires the minimum (only one) nucleotide change. Val is most frequently substituted for by Ile and Ala followed by Leu, Met, and Thr in that order in various proteins without apparent phenotypic change (Jones *et al*., 1992). As a matter of fact, Leu can be found in place of Val in the RRF homologue of *Aquifex aeolicus* (Deckert *et al*., 1998), *Chlamydia trachomatis* (Zhang *et al*., 1997) and *Saccharomyces cerevisiae* (Johnston *et al*., 1994). However, to replace Asp (GAU) in that position by Ile (AUA, AUC, AUU) or Leu (CUA, CUC, CUG, CUU, UUA, UUG) would require two or three mutational changes in the same codon. This may be the reason why we only found the reversion of Asp to Val among our intragenic revertants.

On the other hand, Ala is frequently found in RRF homologues in this position, as in the RRF of the *Synechocystis* (Kaneko *et al*., 1996), *Brucella melitensis* (Vizcaino *et al*., 1996), *Helicobacter pylori* (Tomb *et al*., 1997), *Mycobacterium leprae* (GenBank # 3122778), *Mycobacterium tuberculosis* (Cole *et al*., 1998), *Borrelia burgdorferi* (Fraser *et al*., 1997), *Mycoplasma pneumoniae* (Himmelreich *et al*., 1996) and *Caenorhabditis elegans* (GenBank # 3123156). Therefore, Asp117 (GAT) in ts *frr14* could change to Ala (GCT) with a single nucleotide change. Yet, we did not observe any reversion by Asp117Ala suggesting that the functional integrity of *E. coli* RRF may best be preserved with valine in this position.

Intergenic suppression of ts *frr14*

With all 14 independently isolated intergenic suppressor mutants, the restoration of the growth at 43 °C was not fully complete, resulting in small colonies at the elevated temperature. It has been established that RRF must act together with EF-G (Hirashima & Kaji, 1973) or RF-3 (Grentzmann *et al*., 1998). Since RRF plays a role in preventing translational error (Janosi *et al*., 1996b), it is likely that RRF binds to the A site, the decoding site of the ribosome. Recent kinetic experiments using synthetic mRNA supports this notion (Pavlov *et al*., 1997). It is therefore tempting to speculate that one of the suppressor genes may code for EF-G, or RF-3 or the components of the A-site such as L12/L7 (Agrawal *et al*., 1998; Stark *et al*., 1997). In addition, ribosomal RNA nucleotide 1492-1493, 529-530 of 16 S RNA (Moazed & Noller, 1986) and 2584-2585 of 23 S RNA (Moazed & Noller, 1989; Steiner *et al*., 1988) may be involved. Mutational alterations of these proteins or RNAs could very well be deleterious to the cell, thus explaining the small colony size as discussed above. The intergenic suppression leading to a tr-small strain should include changes in the frame shifted chromosomal *frr* to one which, though not functional by itself, assists in the functioning of ts RRF at 43 °C. The interaction of two non-functional proteins has been reported to produce the activity in some cases.

It is known that RRF has two functions, i.e. to disassemble the post termination complex (Janosi *et al*., 1996a, 1998) and to prevent translational error (Janosi *et al*., 1996b), although these may be two different expressions of the same function. It is possible that, depending on the gene which was mutated for the suppression of ts *frr14*, the suppressor gene may complement one function of RRF, but not the other. It is therefore understandable that these independently isolated suppressors can be classified into at least four different groups (Table 5). Theoretically, there should be at least two types of suppression. One is the direct complementation of the temperature-sensitive RRF function and the other is an indirect suppression *via* reducing the detrimental effect of the nonfunctional RRF. Work is in progress to elucidate the nature of the suppression of the 14 suppressor mutations described in this paper.

Materials & Methods

Strains and plasmids

All bacteria, except for the *E. coli* DH5a strain (BRL/Gibco), are derivatives of *E. coli* MC1061 (Casadaban & Cohen, 1980). The plasmids used in this study are derivatives of pSC101 replicon (Cohen & Chang, 1977). The strain LJ4 (pPEN1560) (Janosi *et al*., 1998) is MC1061 *frr1* (non-functional frame shifted (fs) allele of *frr*, Δ(*srl-recA*) *306*::Tn*10* (*RecA*$^-$ tetracycline (TC) resistant)), harboring plasmid pPEN1560. Plasmid pPEN1560 is a derivative of pSC101 that carries *frr* (the wild-type (wt) allele, with its promoter that resides within 100 nucleotides upstream of the *frr* open reading frame, and its *rho* independent transcription terminator residing within 73 nucleotides downstream from the reading frame in a 0.9 kb *Kpn*1-*Hin*dIII fragment) and the *sacB*-Neo^r cassette (Blomfield *et al*., 1991) encoding sucrose sensitivity and KM resistance. Plasmid pMIX (Janosi *et al*., 1998) consists of a pSC101 replicon, the *cat* gene of pBR325 (Bolivar, 1978) encoding CM resistance and randomly mutated *frr* as the 0.9 kb *Kpn*I-*Hin*dIII insert. Plasmid pPEN907 (Janosi *et al*., 1998) is identical with pMIX except that the 0.9 kb *Kpn*I-*Hin*dIII insert carries wt*frr*. The latter plasmid was used as a template in error prone PCR (Zhou *et al*., 1991) to obtain randomly mutated *frr*. Plasmid pPEN1586 originated from pMIX and carries the ts *frr14*. Plasmid pKH6 was derived from the pMW119 vector (Nippongene), a pSC101 replicon with the AP resistance marker, carrying ts *frr14* as the 0.9 kb *Kpn*I-*Hin*dIII fragment from pPEN1586. Plasmids pKH1, 5, 8, 10 and 16 are identical with pKH6 except that the 0.9 kb *Kpn*I-*Hin*dIII fragment in all of these plasmids carry ts *frr15*, *13*, *4*, *6* and *7* (Janosi *et al*., 1998), respectively.

Media

LB medium was used as liquid medium. LB agar (LA) was prepared from LB by adding 1.5% (w/v) Bacto agar to LB. The sucrose (S) medium is identical with the LA medium except that NaCl is replaced by 6% (w/v) sucrose (Blomfield *et al*., 1991). The media were supplemented with CM (20 μg/ml), KM (50 μg/ml) and AP (100 μg/ml) when needed.

Random mutagenesis of *frr* and DNA techniques

Mutated *frr* was obtained as described (Janosi *et al*., 1998) by making error-prone PCR on the wt*frr* insert of pPEN907, digestion of the PCR product with *Kpn*I and *Hin*dIII restriction endonucleases, and isolating the mutated DNA fragment from agarose gel as a 0.9 kb DNA fragment. Plasmid DNA isolation, restriction endonuclease digestion, agarose electrophoresis and plasmid DNA specific transformation of *E. coli* cells were carried out as described (Sambrook *et al*., 1989).

DNA sequencing of the mutated *frr*

Nucleotide sequencing of ts mutations and intragenic suppressor mutations of ts *frr14* was carried out with automated nucleotide sequencing facilities in Philadelphia on a commercial basis. For analysis of the null mutations, highly automated DNA sequencing facilities of Nara Institute of Science and Technology were used. The DNA sequencer was ABI 377; the sequencing kit was ABI dye terminator; and the universal primer for forward and reverse directions were used.

Classification of intergenic suppressor mutations

The intergenic suppressor strains of *E. coli* temperature-sensitive *frr* mutations, tr-small 1 through 14, harboring pPEN1586 carrying *ts frr14* and CM resistance, were grown at 28 °C overnight in LB broth supplemented with 20 μg/ml of CM. They were separately transformed with the plasmids pKH1, pKH5, pKH8, pKH10, and pKH16 carrying ts *frr15*, *frr13*, *frr4*, *frr6* and *frr7*, respectively, using AP resistance as the transformation marker. Four single colonies of all transformants were taken and passed three times consecutively on LB

plate supplemented with 50 μg/ml of AP at 28 °C to eliminate the resident plasmid pPEN1586 by the plasmid incompatibility principle. The loss of the resident plasmid was confirmed by sensitivity to CM. The transformants harboring the pKH plasmid were cultured on LB plates containing AP 50 μg/ml at 28 °C and 42 °C.

Note added in proof

Our recent data on the structure of RRF of *Themotoga maritima* and *Rsudomonas aeruginosa* by X-ray crystallography and NMR spectroscopy, respectively, are essentially in agreement with the predicted structure shown in Figure 1. (Selmer, M., Al-karadaghi, S., Hirokawa, G., Kaji, A. & Liljas, A. (1999). *Science*, in the press; Kashimori, H., Yoshida, T., Kijima, H., Schimahara, H., Uchiyama, S., Ishino, T., Shuda, M., Nakano, H., Shibata, Y., Saihara, Y., Ohkubo, T., Yoshida, T., Kaji, A. & Kobayashi, Y. (1999). *J. Biomol. NMR*, in the press.)

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