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Molecular Cloning, Sequencing, Purification, and Characterization of *Pseudomonas aeruginosa* Ribosome Recycling Factor

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Ribosome recycling factor (RRF) is required for release of 70S ribosomes from mRNA on reaching the termination codon for the next cycle of protein synthesis. The RRF-encoding gene (*frr*) of *Pseudomonas aeruginosa* PAO1 was functionally cloned by using a temperature-sensitive *frr* mutant of *Escherichia coli* and sequenced. The *P. aeruginosa frr* was mapped at 30 to 32 min of the *P. aeruginosa* chromosome. The deduced amino acid sequence of RRF showed a 64% identity to that of *E. coli* RRF. In an assay including *E. coli* polysome and elongation factor G, purified recombinant RRF of *P. aeruginosa* released monosomes from polysomes. This is the first case in which an RRF homologue was found to be active in heterogeneous ribosome recycling machinery. The genes for ribosomal protein S2 (*rpsB*), elongation factor Ts (*tsf*), and UMP kinase (*pyrH*) are located upstream of *frr*. The arrangement of the genes, *rpsB-tsf-pyrH-frr*, resembles those reported for *E. coli* and *Bacillus subtilis*. Even in the cyanobacterium genome, the arrangement *pyrH-frr* is conserved. Although RRF homologues are found in eukaryotic cells, phylogenetic analysis suggests that they were originally present within the members of the phylogenetic tree of prokaryotic RRF. This finding suggests that the ribosome recycling step catalyzed by RRF is specific for prokaryotic cells and that eukaryotic RRF is required for protein synthesis in organelles, which are believed to be phylogenetically originated from prokaryotes.

Termination of protein synthesis in *Escherichia coli* is catalyzed by the peptide release factors RF1, RF2 and RF3, creating the posttermination complex (5, 31, 42). In the presence of RRF (ribosome recycling factor, previously called ribosome releasing factor), and either the elongation factor EF-G (16) or RF3 (9), the translational posttermination complex is disassembled into mRNA, tRNA, and the ribosome (13, 32). This step is the fourth step of protein biosynthesis. RRF is also involved in the elongation step to assure that cognate aminoacyl tRNA is placed on the ribosomal A site (23). RRF is a basic protein coded for by the gene named *frr*, which has a very strong promoter (39) with minimal expression under labora-

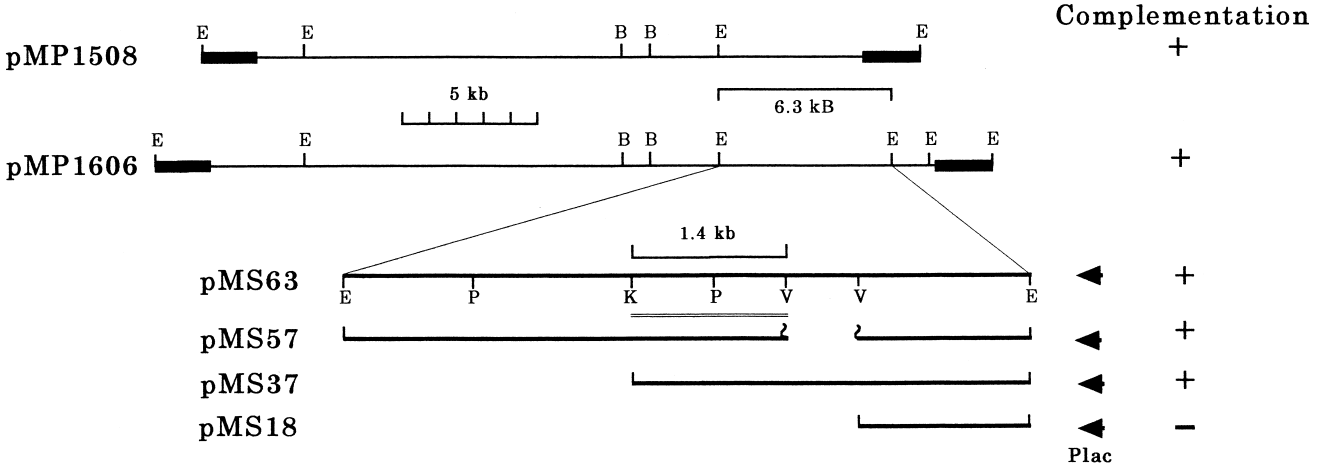

FIG. 1. Restriction maps of *P. aeruginosa* PAO1 chromosomal DNA insert in pMP1508, pMP1606, and subclone pMS63. The bold lines represent regions of vector pLA2917. pMS63 has a 6.3-kb *Eco*RI fragment from pMP1606. The results of complementation experiments with various plasmids are indicated to the right of the restriction maps. Restriction sites are as follows: B, *Bgl*II; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; V, *Eco*RV. The complementation experiment was done as follows. Each plasmid was transformed into LJ1036, and then transformants were selected on Ap-containing plates. Forty-five transformants of each plate were streaked on Ap-containing plates and incubated at 32 and 43°C. + indicates that all transformants grew at both 32 and 43°C, and − means that all transformants grew at 32°C but not at 43°C.

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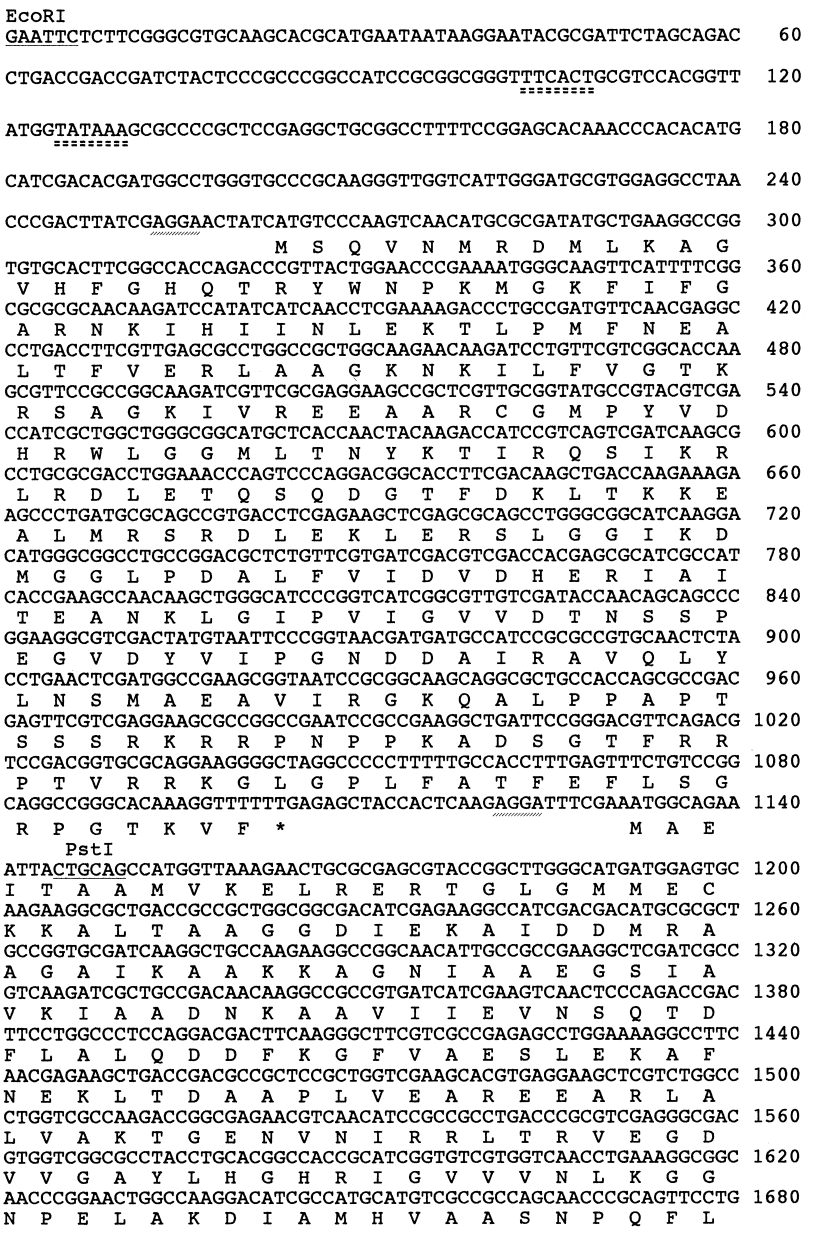

FIG. 2. DNA sequence of a 4,003-bp region containing *rpsB*, *tsf*, *pyrH*, and *frr* genes. The deduced amino acid sequences are shown below the DNA sequences. Potential promoters and ribosomal binding sites are indicated as double broken lines and hatched underlines, respectively. Transcriptional terminators are indicated by dots. *Eco*RI, *Pst*I, *Kpn*I, and *Eco*RV sites are underlined.

tory conditions (20) but elevated expression in some pathogens that infect animals (29, 44). RRF is an essential for *E. coli* (21) and is present in every type of prokaryote (23) except for archaebacteria (*Archaea*). (For more information on RRF see recent reviews in references 22, 23, and 25.)

Until recently studies on RRF were carried out almost exclusively on *E. coli* RRF. The fact that *frr* is essential (21) and the wide distribution (23) of its homologues in nature strongly suggest the biological importance of RRF. Structural and functional studies of the *frr* genes and RRF protein from diverse species will give useful information on both the RRF proteins themselves and the ribosome recycling step as a possible target of antimicrobial agents. In this paper, we show that an *frr* homologue of *Pseudomonas aeruginosa* (frr_{PA}) complements functionally the *E. coli frr* (frr_{EC}) mutant. Furthermore, the purified RRF was found to be active in an in vitro assay system including *E. coli* ribosomes and EF-G, though its activity in *E. coli* is lower than that of *E. coli* RRF (ECRRF).

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and culture conditions. The list of plasmids and strains used is provided in Table 1. Temperature-sensitive strains were grown at 32°C or, for complementation assay, at 43°C. Luria-Bertani (LB) broth (Difco, Detroit, Mich.) was used in liquid and solid agar (1.5%) media for routine cultivation of bacteria. In some cases, the media were supplemented with antibiotics (50 μg of ampicillin [Ap] per ml, 20 μg of tetracycline [Tc] per ml, 20 μg of chloramphenicol [Cm] per ml, and/or 500 μg of streptomycin [Sm] per ml).

Construction of genomic library and DNA manipulation and sequencing. Genomic DNA library of *P. aeruginosa* PAO1 was constructed by using the

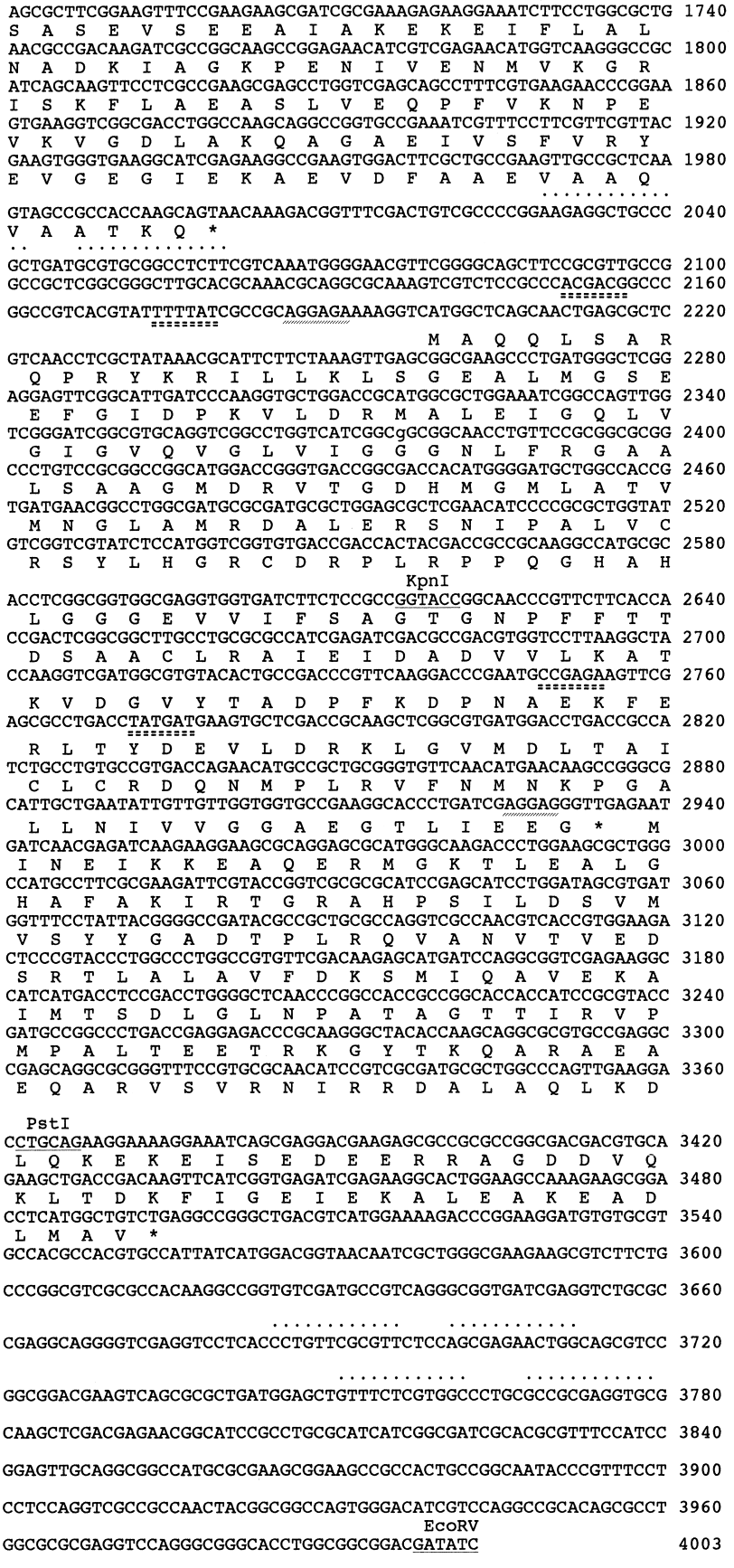

FIG. 2—*Continued.*

TABLE 1. Strains and plasmids

Name	Characteristics[a]	Source or reference
Strains		
P. aeruginosa PAO1	Prototrophic	17
E. coli		
S17-1	*pro thi recA hedR*; RP4-2 *trf*$^+$ TC::Mu Km::Tn7 AP::ISR1	40
MC1061	F$^-$ *frr*(Wt) *araD139 ara-leu7679* (*lacIPOZYA*)*X74 galU galK hsdR2 mcrB1 rpsL* (Sm^r)	21
LJ14	MC1061 *frr14*(Ts)	24
LJ3	MC1061 *frr1*(Fs) Δ(*sr1-recA*) *306*::Tn*10*[b]	24
LJ1036	LJ3 transformed with pPEN1944 (carries Ts RRF)	This study
LJ2221	LJ14 (F′ *proAB lacI*^{q}ΔM15 Tn*10*)[c]	This study
DH5α	F$^-$ ϕ80d *supE44* Δ*lacU169* (f80 *lacZ*ΔM15) *hsdR17 recA1 gyrA96* F$^-$ ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoP recA1 endA1 hsdR17 phoA thi-1 relA1*	Gibco-BRL
JM109	*recA1 supE44 endA1 hsdR17 tyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	46
Plasmids		
pUC18	Multicopy cloning and sequencing vector; confers Ap^r	46
pUC19	Multicopy cloning and sequencing vector; confers Ap^r	46
pLA2917	Cosmid vector with a broad host range (IncP1) mob$^+$ 21 kb; confers Tc^r	1
pPH1JI	IncP group conjugative plasmid; confers Cm^r	15
pMW118	Ap^r pSC101 derivative cloning vector	Nippongene
pMP1508	pLA2917 with *frr* region of *P. aeruginosa* (see Fig. 1)	This study
pMP1606	pLA2917 with a larger *frr* region of *P. aeruginosa* than pMP1508 (see Fig. 1)	This study
pMS63	pMW118 carrying 6.3-kb *Eco*RI fragment from pMP1606	This study
pMS57	pMS63 with 0.6-kb *Eco*RV fragment deleted	This study
pMS37	pMS63 with 2.6-kb *Kpn*I fragment deleted	This study
pMS18	pMS63 with 4.5-kb *Eco*RV-*Sma*I fragment deleted	This study
pMO2925	pMW118 carrying PCR-amplified *frr* fragment (2925-4003)[d] with *lac* promoter	This study
pSP1814	pUC18 carrying 1.4-kb *Kpn*I-*Eco*RV fragment	This study
pSP1914	pUC19 carrying 1.4-kb *Kpn*I-*Eco*RV fragment	This study
pMAK705	Ts pSC101 replicon; confers Cm^r	10
pPEN1944	pMAK705 carrying *frr14* as a 0.9-kb *Kpn*I-*Hin*dIII fragment; confers Cm^r	This study
pRR2	pUC19 derivative carrying the *frr* gene of *E. coli*	39

[a] Abbreviations: Fs, frame-shift; Ts, temperature sensitive; Wt, wild-type; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance.
[b] 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).
[c] The F′ has been transferred from the XL1 Blue MRF′ strain (Stratagene) by transconjugation.
[d] Nucleotide numbers correspond to *frr* region (see Fig. 2).

cosmid vector pLA2917 (1) and *E. coli* S17-1 (40) as described (30). Preparation of plasmid DNA, agarose gel electrophoresis, and transformation were performed by standard methods (38). DNA sequencing was done by an automated DNA sequencer (model 370A; Applied Biosystems) with fluorescent dye primer supplied by the manufacturer. The DNA sequence was analyzed with GENETYX software (Software Development Co., Tokyo, Japan). The FASTA program and the malign program were used for the homology search of the amino acid sequences and for multiple alignment of homologues and construction of a phylogenetic tree, respectively, through the DNA Data Bank of Japan.

Complementation assay. A temperature-sensitive *frr* mutant of *E. coli*, LJ14 (24), was used as a recipient for conjugation. Conjugal mating was performed as follows. An overnight culture of the recipient cells was spread onto LB plates containing Sm (selects for the recipient) and Tc (selects for the library cosmid). Five microliters of an overnight culture of the donor cells was spotted onto the plates, which were incubated at 43°C. Matings with LJ1036, another temperature-sensitive strain, were carried out in like manner. Transconjugants were selected by resistance to Sm and Tc at 43°C. For the isopropyl-β-D-thiogalactopyranoside (IPTG)-induced complementation assay, overnight culture of LJ2221, which is an LJ14 derivative with *lacI*^{q} on its chromosome, was diluted at 32°C with 40 ml of LB broth with or without 1% glucose to an optical density at 600 nm (OD_{600}) of 0.05 to 0.06 and grown at 43°C with shaking at 120 rpm in baffled flasks with or without IPTG induction (final concentration, 1 mM). Cell growth was monitored by a spectrophotometer reading at 600 nm.

Construction of plasmids. Fragments generated by digestion of pMP1606 with *Eco*RI were cloned into pMW118. Plasmid pMS63 had a 6.3-kb *Eco*RI fragment containing frr_{PA}. The pMS derivatives pMS57, pMS37, and pMS18 were constructed by digesting pMS63 with *Eco*RV, *Kpn*I, and *Eco*RV and *Sma*I, respectively, and ligated by using a ligation kit (Takara, Japan). For pMO2925, we amplified the region extending from nucleotide 2925 to 4003 by PCR using primer 1 (5′-GCGGTACCGAGGAGGGTTGAGAATGATC) and primer 2 (5′-CGGGATCCGATATCGTCCGCCGCCAGGT). The amplified fragment was digested with *Kpn*I and *Bam*HI and was inserted into a region between the *Kpn*I and *Bam*HI restriction sites of pMW118. The primers were obtained from Bio-Synthesis, Inc. (Lewisville, Tex.).

Physical mapping. Southern blot analysis was performed with nonradioactive enhanced chemiluminescence nucleic acid labeling and detection system (Amersham). Pulsed-field gel electrophoresis (PFGE) was carried out with the contour-clamped homogeneous electric field mapper (Bio-Rad) as described (34). The *Spe*I-digested chromosomal DNA was electrophoresed on an agarose gel

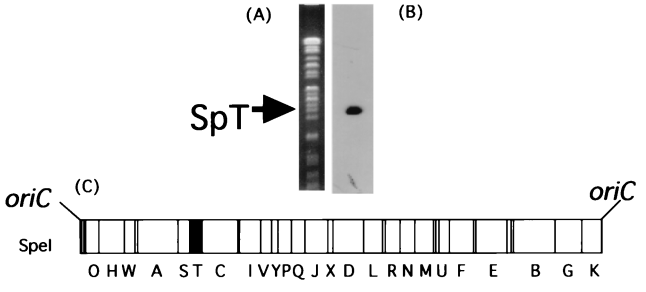

FIG. 3. Physical mapping of *frr* in the *P. aeruginosa* chromosome. (A) Digests of the *P. aeruginosa* PAO1 chromosome with restriction enzyme *Spe*I were separated by PFGE. An arrow indicates the fragment detected by the *frr* probe. (B) Southern blot analysis. The probe used was an 1.4-kb *Eco*RV fragment which contains the complete *frr* gene. (C) Physical map of the genome of *P. aeruginosa*. The SpT fragment (T fragment in the *Spe*I digest of the *P. aeruginosa* chromosomal DNA), which hybridized with the 1.4-kb *frr* probe, is indicated by a closed box.

(1%) and transferred onto a nylon membrane (GeneScreen Plus; New England Nuclear Corp.) under alkaline conditions (38). Hybridization and stringent wash conditions were as follows. After incubation in the prehybridization buffer at 60°C for several hours, a probe was added and incubation was continued at 60°C overnight. The nylon membrane was washed under stringent conditions at 60°C for 15 min with 1× SSC (0.15 M sodium chloride plus 15 mM trisodium citrate [pH 7.0]) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and then washed with 0.5× SSC and 0.1% SDS for 15 min.

Expression of frr_{PA}. *Kpn*I-*Eco*RV 1.4-kb fragments were inserted between the *Kpn*I and *Hin*cII sites of pUC18 and pUC19, yielding pPS1814 and pPS1914, respectively. *E. coli* DH5α harboring pPS1814 and pPS1914 was grown in LB broth containing Ap for 12 h at 37°C, and the cells in 1 ml of culture were collected and disrupted by sonication. After removal of cell debris by centrifugation (15,000 × *g*, 15 min, 4°C), 5 μg of crude extracts was subjected to electrophoresis in an SDS–13% polyacrylamide gel (28) followed by Coomassie brilliant blue staining.

Purification of PARRF and its in vitro assay. With the crude extract of DH5α harboring pPS1814, ammonium sulfate fractionation, DEAE-cellulose column chromatography, Sephadex G-100 column chromatography, and carboxymethyl cellulose column chromatography were performed as described previously (14). Because of the presence of a large amount of *P. aeruginosa* RRF (PARRF) in this strain, the carboxymethyl cellulose-Sephadex step described previously (14) was not necessary. The assay of RRF was performed with puromycin-treated *E. coli* polyribosomes as a substrate, as described previously (11). The preparation of polyribosomes to be used as the substrate for RRF may contain a substantial amount of monosomes, but this does not influence the assay (11). The RRF activity in this assay system is measured as the amount of polysomes converted to monosomes, expressed as the percentage of the total ribosomes in the reaction mixture. For example, if RRF converted to monosomes all polysomes of a preparation consisting of 30% polysomes and 70% monosomes, the RRF activity is expressed as 30% conversion. In this case, the reaction of RRF is complete, because all the available polysomes were converted to monosomes.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB010087.

RESULTS

Cloning of *frr* homologue from *P. aeruginosa* library by complementation. *P. aeruginosa* and *E. coli* belong to the same group, proteobacteria, gamma subdivision. We therefore assumed that *P. aeruginosa* RRF can complement *E. coli* LJ14, which has temperature-sensitive RRF. By using *P. aeruginosa* genomic cosmid library (30), two plasmids, pMP1508 and pMP1606, were found to convert LJ14, and LJ1036 (*recA* of LJ14) to strains with wild-type phenotype. Segregation of the plasmid reverted these strains to temperature sensitive.

The DNAs of pMP1508 and pMP1606 were digested with *Eco*RI and *Bgl*II, and generated fragments were subcloned into the *Eco*RI site of pMW118 and pMS63 as shown in Fig. 1. All of the transformants of LJ1036 selected at 43°C contained pMS63 carrying a 6.3-kb fragment insert. To further locate the complementing gene, a series of deletions was introduced into pMS63 by digestion with various enzymes, including *Eco*RV and *Kpn*I. Plasmids pMS57 and pMS37 but not pMS18 could complement, suggesting that the *frr* gene is located in the 1.4-kb fragment between the *Kpn*I and *Eco*RV sites shown as a double line in Fig. 1.

Nucleotide sequence of DNA fragment containing *frr* and physical mapping. The sequence of the 4.0-kb *Eco*RI-*Eco*RV fragment in pMS57 was determined (Fig. 2). Four major open reading frames (ORFs) were found within this fragment. The fragment contained 62.5% G+C content, and about 80 to 85% of the third positions of the codons of these ORFs were G or C. These values are in agreement with the reported G+C content and the G+C frequency in the third position of *P. aeruginosa*.

Within the *Kpn*I-*Eco*RV fragment, one complete ORF, at nucleotides 2939 to 3496, was noted. The ORF is preceded by a putative promoter and a ribosome binding sequence (AGGAG) complementary to the 3′ end of the 16S rRNA of *P. aeruginosa*, 3′-AUUCCUCU (7). Potential stem-loop structures exist downstream of the ORF (at nucleotides 3685 to 3712 and 3751 to 3779); however, the structure was far apart from the termination codon and is not followed by a poly(A) sequence. This ORF encoded a protein of 185 amino acid residues, and showed an extended homology to ECRRF (19), with 64.5% identity.

The deduced polypeptides from the other three ORFs upstream of *frr* (Fig. 2) showed homology with ribosomal protein S2 (*rpsB*) (nucleotides 263 to 1105), elongation factor Ts (*tsf*) (nucleotides 1132 to 2001), and UMP kinase (*pyrH*) (nucleotides 2199 to 2936). Downstream of *frr*, the *Pst*I-*Eco*RV region completely matched with the upstream sequence of *cdsA*, which encodes CDP-diglyceride synthetases described by Taguchi et al. (41).

A 1.4-kb *Kpn*I-*Eco*RV fragment (indicated by a double line below pMS63 in Fig. 1) hybridized with a 120-kb fragment, designated SpT (18), of PAO1 DNA (Fig. 3). A 1.2-kb *Pst*I-*Eco*RI fragment also hybridized with SpT (data not shown). This finding suggests that these four genes, *rpsB*, *tsf*, *pyrH*, and *frr*, are located within 30 to 32 min on the *P. aeruginosa* chromosome. This is consistent with the fact that genes for biosynthetic pathways and housekeeping functions of *P. aeruginosa* cluster in the auxotroph-rich region that spans 60% of the chromosome, from SpB to SpI (18).

Comparison of deduced amino acid sequences of RRF homologues of eubacteria. The *frr* genes of several species have already been sequenced, although the functions of their gene products except ECRRF have not yet been analyzed. Shown in Fig. 4 are sequences of *frr* genes from the following four divisions of eubacteria: proteobacteria (alpha subdivision, *Brucella melitensis* [44]; gamma subdivision, *E. coli* [19] and *Haemophilus influenzae* [6]; epsilon subdivision, *Helicobacter pylori* [43]), planctomycetales (*Chlamydia trachomatis* [U60196]), firmicutes (actinomycetes, *Mycobacterium tuberculosis* [Z74024] and *Mycobacterium leprae* [L78824]; low-G+C-content gram-positive bacteria, *Bacillus subtilis* [27], *Mycoplasma genitalium* [8] and *Mycoplasma pneumoniae* [12]), and cyanobacteria (*Synechocystis* sp. PCC6803 [26]). In addition to these eubacterial *frr* homologues, eukaryotic homologues of higher plants, *Daucus carota* (X72384) and *Spinacia oleracea* (37), and of yeast (34) were also aligned. The deduced eubacterial RRF molecules are of similar sizes (ranging between 179 and 186 amino acids), while eukaryotic homologues had additional peptides in the N-terminal portion. However, a high degree of amino acid sequence conservation allows alignment of nearly the entire length of the molecule with minimal ambiguity except for their N-terminal regions. In this alignment four amino acid residues, proline 103, arginine 110, lysine 115, and lysine 178, were completely conserved, suggesting that these residues were essential for RRF function. The Ts mutant, *frr14*, has a mutation resulting in replacement of the valine at 117 with aspartic acid (24). All amino acid residues of the homologues at the position corresponding to 117 of ECRRF were hydrophobic residues. The hydrophobicity of the amino acid residues at this position may therefore be required for maintenance of RRF stability.

RRF of *P. aeruginosa* is functional in *E. coli*. To further confirm whether the cloned frr_{PA} codes for functional RRF, we constructed pMO2925, which carries the fragment bearing frr_{PA} without its own promoter but with the *lac* promoter. As shown in Fig. 5, pMO2925 could complement temperature-sensitive growth of LJ2221 carrying temperature-sensitive *frr* when 1 mM IPTG was added, while without IPTG the growth of the cells at 43°C was restricted. Thus, the product translated from frr_{PA} was functional in *E. coli*. The cell density of LJ2221 (pMO2925) slowly increased in the absence of IPTG. As the growth was not suppressed by the presence of glucose, some

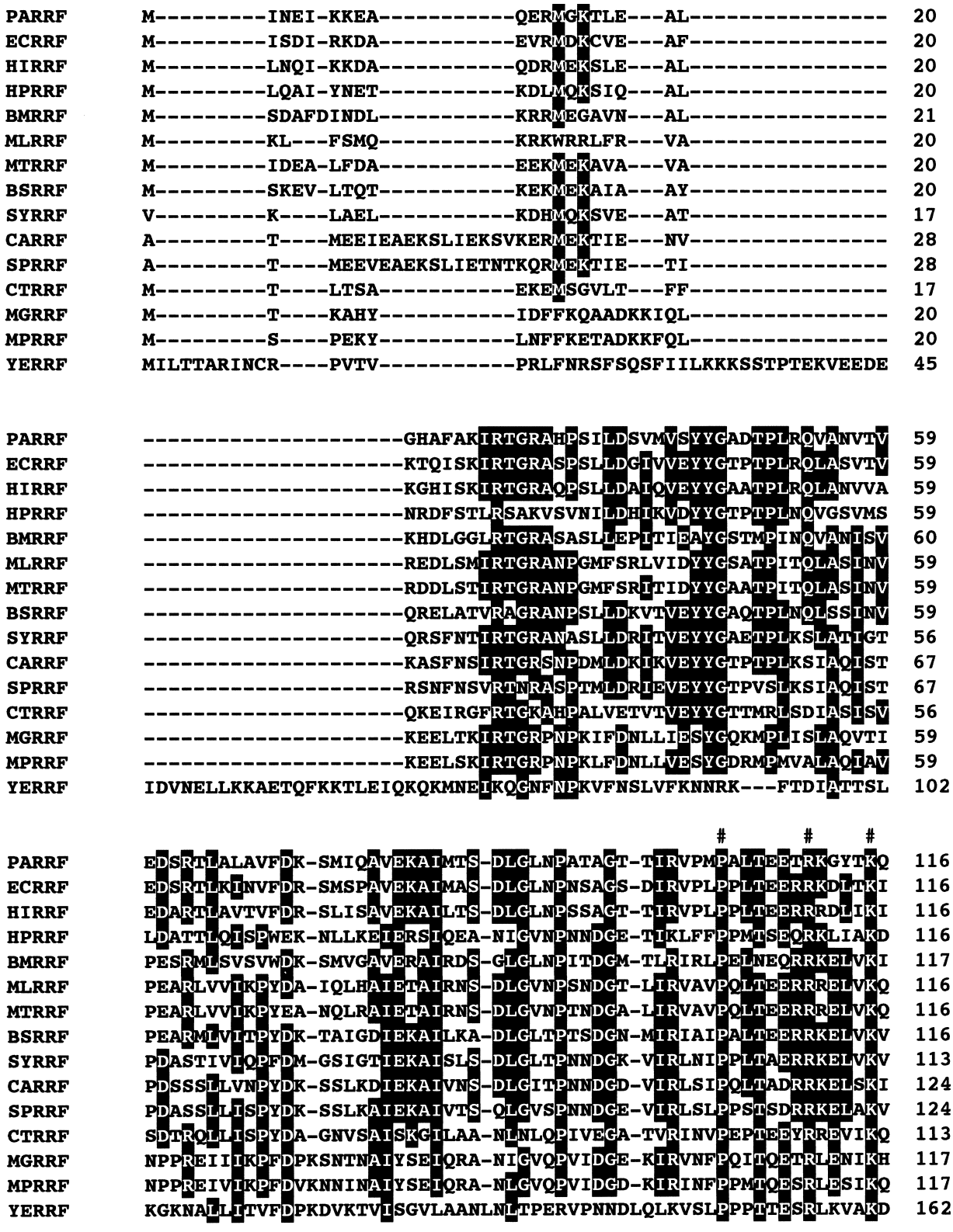

FIG. 4. Comparison of the deduced amino acid sequences of RRF. The malign program was used for the comparison (11). Gaps, indicated by dashes representing empty positions, are introduced in order to obtain a maximum fit. Total numbers of residues are indicated on the right. PA, *P. aeruginosa*; EC, *E. coli* (19); HI, *H. influenzae* (6); HP, *H. pylori* (43); BM, *B. melitensis* (44); ML, *M. leprae* (accession no. L78824); MT, *M. tuberculosis* (accession no. Z74024); BS, *B. subtilis* (27); SY, *Synechocystis* sp. strain PCC6803 (24); CA, *D. carota* (accession no. X72384); SP, *S. oleracea* (37); CT, *C. trachomatis* (accession no. U60196); MG, *M. genitalium* (8); MP, *M. pneumoniae* (12); YE, yeast (34). Black boxes indicate positions conserved in at least 51% of the aligned sequences. # indicates that the amino acids are identical in all sequences.

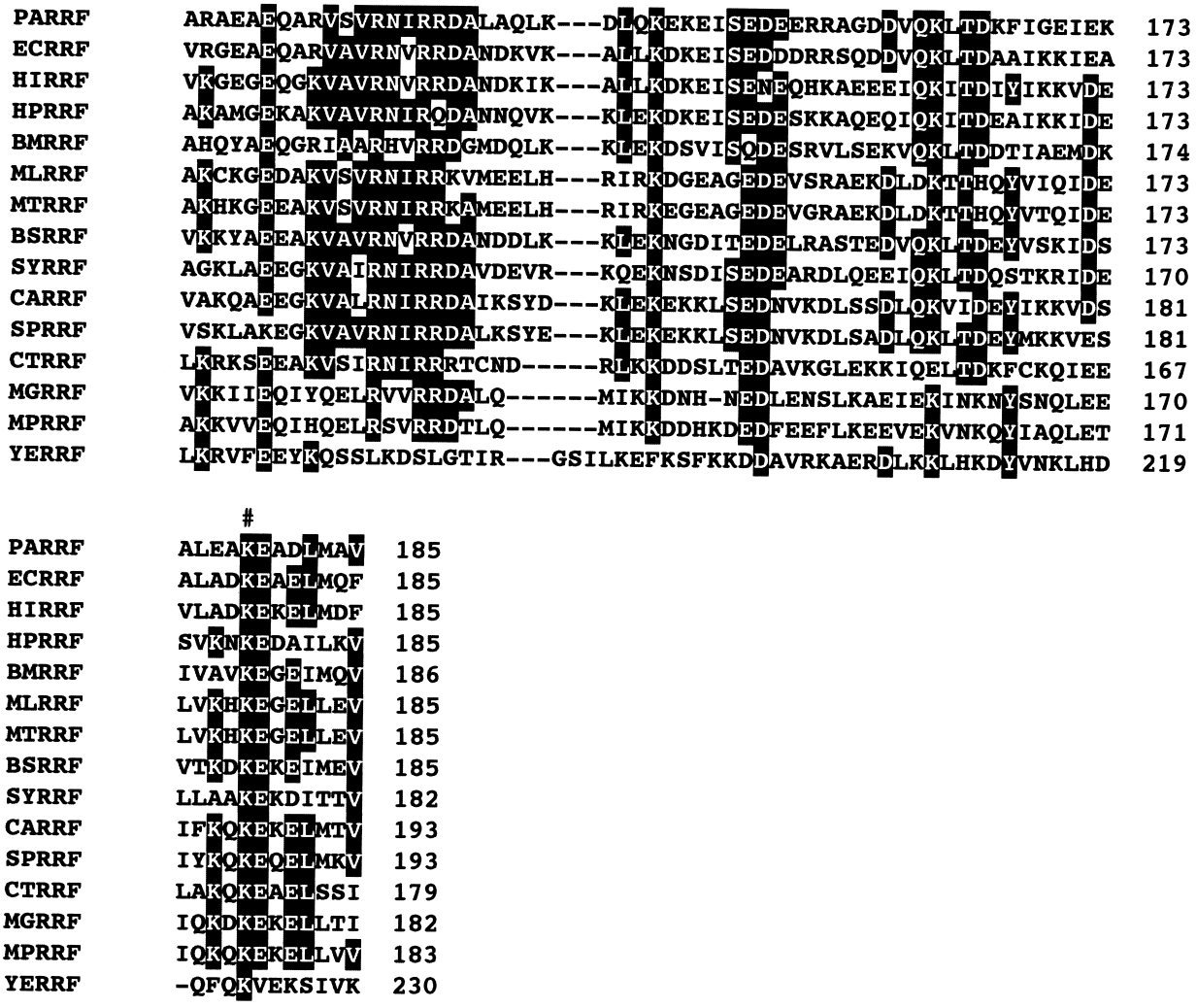

FIG. 4—*Continued.*

residual promoter activity from the vector sequence may allow the expression of frr_{PA} in *E. coli* to some extent.

Purification of recombinant PARRF and its ribosome recycling activity in vitro. To develop an expression system for PARRF, we constructed a pUC18 recombinant, pPS1814, containing the *Kpn*I-*Eco*RV 1.4-kb fragment (see pMS63, Fig. 1), in which *frr* was placed in the same direction with the *lac* promoter. As shown in Fig. 6, *E. coli* DH5α cells harboring pPS1814 produced a large amount of PARRF without induction by IPTG. ECRRF (lane 4) migrated slightly slower than PARRF (lane 2). pPS1914, which has the frr_{PA} in the opposite direction to the promoter (lane 3), did not produce PARRF in visible quantity (Fig. 6).

PARRF was purified from DH5α harboring pPS1814 by a modified simple purification procedure, essentially by the original method for purification of ECRRF (16). As shown in Fig. 7, the purified PARRF cross-reacted with polyclonal anti-ECRRF antibody, although approximately 30-fold more *E. coli* antibody was required to obtain an equivalent reaction with PARRF.

To confirm that PARRF could actually release ribosomes from mRNA, we carried out the in vitro assay for the RRF activity using puromycin-treated *E. coli* polysomes and EF-G. The purified PARRF converted the polysomes into monosomes, suggesting that PARRF released the ribosomes from the mRNA-ribosome complex with the aid of *E. coli* EF-G (Fig. 8). However, PARRF was not as active as in this assay including *E. coli* polysomes. This confirmed our conclusion, derived from data presented in Fig. 5, that PARRF does not function in *E. coli* as well as ECRRF.

DISCUSSION

In this paper, we present the first detailed characterization of RRF homologue from a pathogen, *P. aeruginosa*. The frr_{PA} was found to function in *E. coli*; this is the first demonstration that another bacterial RRF homologue can complement temperature-sensitive *E. coli* strain LJ14 carrying temperature-sensitive RRF (24). In view of the failure of complementation of LJ14 by other RRF homologues, including those of *Staphylococcus aureus*, *H. pylori*, *Streptococcus faecalis*, and *Thermotoga maritima* (unpublished observation), the success of complementation of LJ14 by PARRF is a unique case.

The gene arrangement of the region spanning *rpsB*, *tsf*, *pyrH*, and *frr* is entirely conserved in *P. aeruginosa*, *E. coli*, and *B. subtilis* (Fig. 9). The genes *rpsB* and *tsf* are components of the

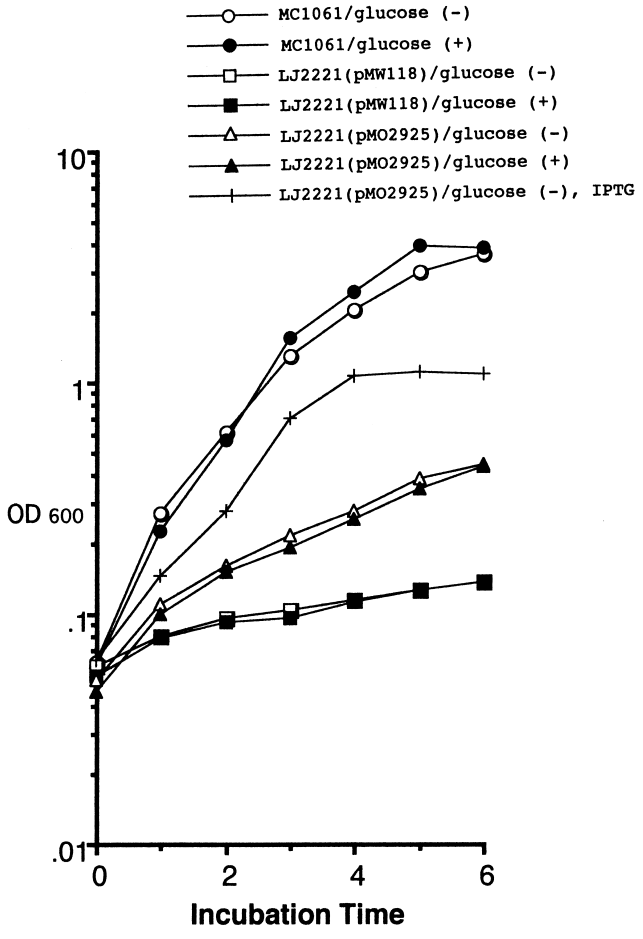

FIG. 5. Growth at elevated temperature of *E. coli* MC1061 (with wild-type *frr* in the chromosome), its *frr*(Ts) mutant derivative LJ2221, and LJ2221 with a vector plasmid (pMW118) containing frr_{PA} under the control of the *lac* promoter. LJ2221 harboring pMO2925 was grown overnight at 32°C. At time 0, the overnight culture was diluted in LB broth (OD_{600} of 0.05) and incubated at 43°C, and the OD_{600} was plotted against the time (h) after the temperature shift. The growth curves are obtained from a representative experiment. Conversion of the temperature-sensitive LJ2221 to a strain which is temperature-resistant by frr_{PA}, as indicated in this figure, was confirmed repeatedly.

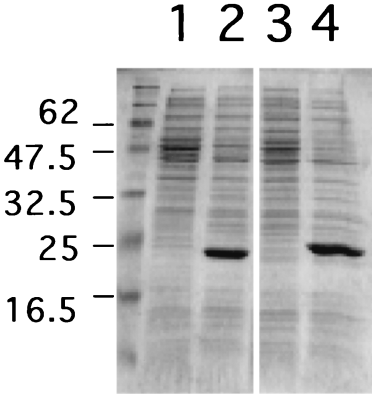

FIG. 6. Expression of PARRF in *E. coli* DH5α. Cytosoluble proteins (5 μg) were separated on an SDS-polyacrylamide gel, and the gel was stained by Coomassie brilliant blue R-250. Lanes: 1, DH5α (pUC18); 2, DH5α (pSP1814); 3, DH5α (pSP1914); 4, DH5α (pRR2). Plasmids pSP1814 and pSP1914 are pUC18 and pUC19 carrying the 1.4-kb frr_{PA} fragment, respectively. The direction of the promoter of the latter is opposite to that of the former. pRR2 is pUC19 carrying the frr_{EC} gene.

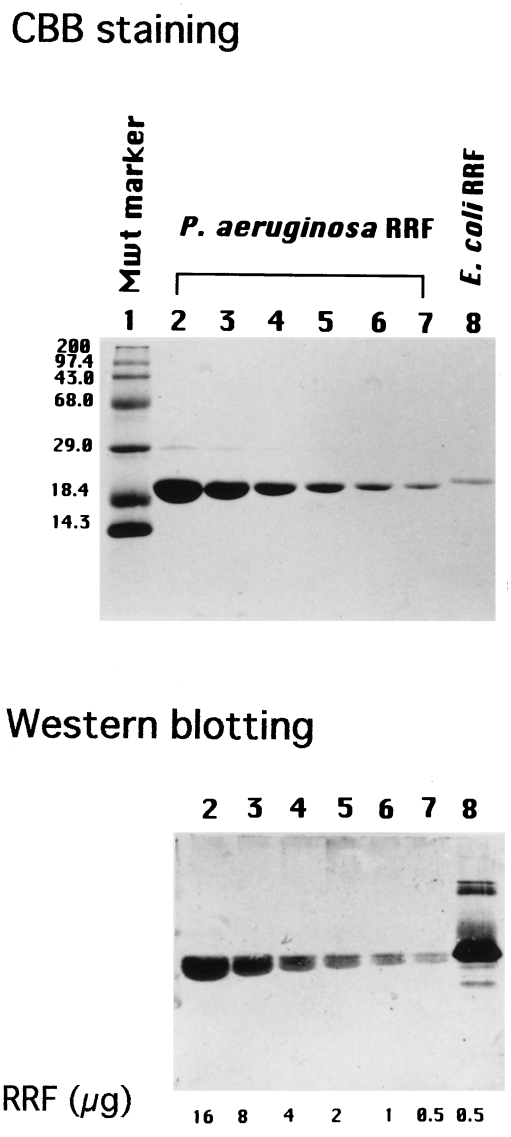

FIG. 7. Antigenic cross-reactivity of *P. aeruginosa* RRF with antibody to *E. coli* RRF. The purified RRF was separated by SDS-polyacrylamide gel electrophoresis. Lane 1 (for Coomassie brilliant blue [CBB] staining) shows molecular weight (Mwt) markers. Lanes 2 to 7 show the *P. aeruginosa* RRF which was twofold serially diluted from 16 to 0.5 μg. Lane 8 contains 0.5 μg of *E. coli* RRF. In the Western blotting experiment, rabbit antibody against *E. coli* RRF diluted 20,000-fold was reacted with the purified *P. aeruginosa* and *E. coli* RRF on nitrocellulose membrane transferred from the gel after electrophoretic separation as described above. Since PARRF was expressed in *E. coli* when the purified enzyme was diluted, two bands are visible. The upper band is that of ECRRF. Since ECRRF is much more reactive with the antibody, the amount of PARRF is about 30 times more than that of ECRRF even though these two bands have almost equal density in the Western blotting.

translational apparatus, and in *E. coli*, both genes form a single transcriptional unit (2). In *P. aeruginosa* these two genes may form a polycistronic structure, because a putative promoter is located upstream of *rpsB* and *rpsB* is followed by *tsf* with a distance of 26 bp (Fig. 2). It should be noted that the *pyrH-frr* gene arrangement is conserved even in *Synechocystis* sp. The observed conservation of the gene order supports the concept that *frr* evolved at an early stage in the evolution of eubacteria. Although *H. influenzae* and *H. pylori* are more closely related to *P. aeruginosa* and *E. coli* than to *B. subtilis* and *Synechocystis*

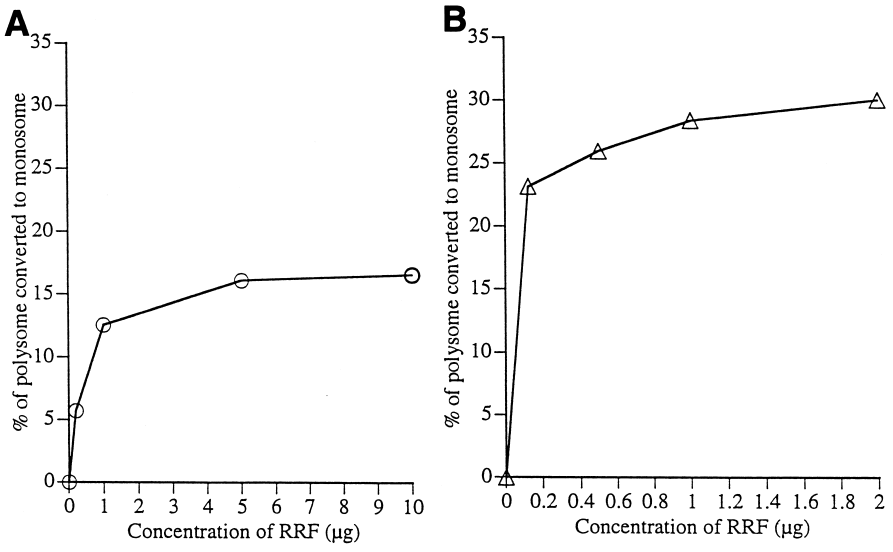

FIG. 8. Comparison of specific activity of purified *P. aeruginosa* RRF with that of *E. coli* RRF. (A) *P. aeruginosa* RRF and (B) *E. coli* RRF. The reaction mixture (275 μl) contained 10 mM Tris (pH 7.4), 80 mM NH_4Cl, 8.2 mM $MgSO_4$, 1 mM dithiothreitol, 10 μM puromycin, 160 μM GTP, polysomes (OD_{260} of 8.0), and 94 μg of S150 free of RRF. Various amounts of RRF were added to the reaction mixture (as indicated) and incubated for 15 min at 30°C. After incubation, 0.275 ml of reaction mixture was placed on the top of a 5-ml linear sucrose gradient (15 to 30%) in buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 10 mM $MgSO_4$, and 0.5 mM dithiothreitol and centrifuged at 40,000 rpm for 1 h in a Beckman SW50.1 centrifuge at 4°C. The profile of the polysome fraction was obtained by the ribosomal sedimentation profile at OD_{254} (ISCO apparatus). Then, the values for conversion of the polysomes to monosomes, calculated as percentages of the total ribosomes, due to the addition of different amounts of RRF were plotted. The amounts of polysomes available for conversion were 21.69% and 36.46% (expressed as percentages of the total ribosome count) for panels A and B, respectively. The low activity of PARRF relative to that of ECRRF, as shown in this figure, has been confirmed repeatedly.

sp., the *pyrH-frr* arrangement is not conserved in *H. influenzae* and *H. pylori* genomes, suggesting that rearrangement of genes may frequently occur in the genomes of these bacteria.

As shown in Fig. 10, comparison of RRF sequences from various organisms suggests that the gene coding for RRF phylogenetically originated in prokaryotes, entered into ancestral eukaryotes as a part of organelles, and became incorporated into the chromosome. Therefore, it is expected that eukaryotic RRF is localized in organelles. Indeed, the plant RRF is found in chloroplasts, the photosynthetic organelle (unpublished observation). It should be noted that RRF of the most primitive eukaryote, yeast, shares a direct ancestor with RRF of the most primitive prokaryote, *M. genitalium*, the smallest free-living organism and the most primitive prokaryote (Fig. 10). The

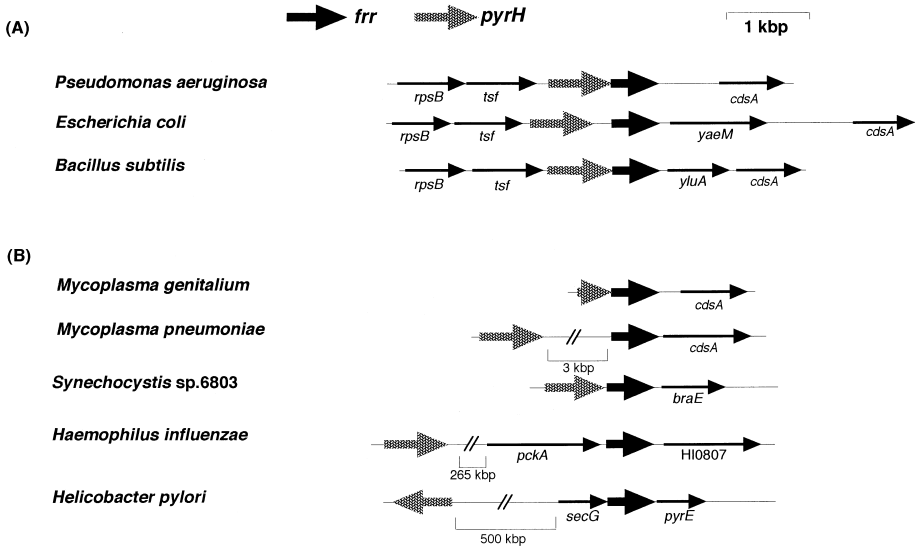

FIG. 9. Comparison of gene arrangements of the regions containing *frr* homologues. Complete conservation of arrangement of the sequence *rpsB-tsf-pyrH-frr* (A) and partial conservation or nonconservation of the arrangement (B) are shown. In this figure, we show the arrangements in the species for which whole genome sequences have been published. The regions shown in panel B focus on the region surrounding *frr* and the location of *pyrH*. The depictions of the lengths of the genes and the distances between them are based on the data acquired in this study and those reported for *P. aeruginosa* (41), *E. coli* (3), *B. subtilis* (27), *M. genitalium* (8), *M. pneumoniae* (12), *Synechocystis* sp. (26), *H. influenzae* (6), and *H. pylori* (43).

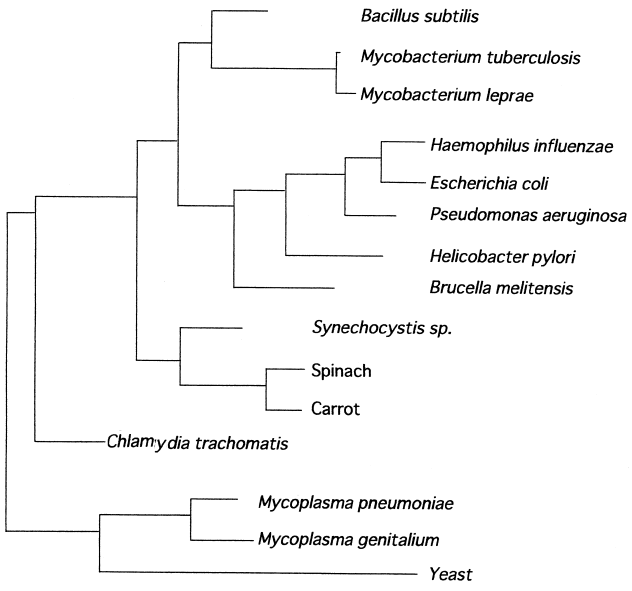

FIG. 10. Phylogenetic tree of prokaryotic and eukaryotic RRF. The tree for the RRF homologue amino acid sequences was calculated by the malign program (11). The horizontal branch lengths are proportional to the differences between sequences.

relationships among the organisms listed in Fig. 10 constructed by ribosomal RNA sequencing revealed a quite different pattern (33). Thus, Fig. 10 suggests the importance of RRF for prokaryotes, while eukaryotic RRF may be important only for maintenance of organelles which are apparently originated from prokaryotes.

The bactericidal and bacteriostatic effects of removal of RRF in vivo suggest that RRF could be a target of antibacterial agents. As suggested by the data shown in Fig. 10, RRF homologue in eukaryotes may be derived from prokaryotes and important only for the maintenance of the organelles. In support of this notion, it is noteworthy that the yeast RRF homologue is not essential because the strain without the *frr* homologue grows well in glucose (45). It appears, therefore, that inhibition of eukaryotic RRF should not influence eukaryotes drastically. In fact, widely used antibiotics, such as Tc, do not have serious side effects even though they inhibit mitochondrial protein synthesis (4, 35).

Inhibition of RRF holds special promise as a possible new way of controlling pathogens. The expression of RRF appears to be very elevated upon infection of animals by *S. aureus* (29). Furthermore, in animals infected with *B. melitensis*, the level of antibody against RRF homologue is extremely elevated. *P. aeruginosa*, a gram-negative opportunistic pathogen, causes severe hospital-acquired infections. This bacterium is well known for its intrinsic resistance to a wide range of antibiotics. The amino acid sequence of PARRF showed a high homology with that of ECRRF. In spite of the homology, both the activity of PARRF in the in vitro system and the reactivity of PARRF with anti-ECRRF antibody were lower than those of ECRRF. These findings imply the existence of a structural difference between PARRF and ECRRF. These considerations strongly suggest that a new drug rationally designed for specific activity against any bacterial RRF may prove to be a very specific means of controlling that particular bacterium without influencing other, innocuous, bacterial flora present in patients.

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