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Role of HPF (Hibernation Promoting Factor) in Translational Activity in *Escherichia coli*

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During the stationary phase of growth in *Escherichia coli*, ribosome modulation factor (RMF) and hibernation promoting factor (HPF) dimerize most 70S ribosomes to form 100S ribosomes. The process of 100S formation has been termed 'ribosomal hibernation'. Here, the contributions of HPF to 100S formation and translation were analysed *in vitro*. HPF bound to, but did not dimerize the 70S ribosome. RMF dimerized and formed immature 90S ribosomes. Binding of both HPF and RMF converted 90S ribosomes to mature 100S ribosomes, which is consistent with the *in vivo* data. The role of HPF in *in vitro* translation also was investigated. In an artificial mRNA poly (U)-dependent phenylalanine incorporation assay, HPF bound to ribosomal particles and inhibited translation. In contrast, in a natural MS2 mRNA-dependent leucine incorporation assay, bound HPF was removed and hardly inhibited normal translation. Multiple alignment and phylogenetic analyses indicates that the hibernation system mediated by the HPF homologue, RMF and 100S ribosome formation may be specific to the proteobacteria gamma group. In contrast, most bacteria have at least one HPF homologue, and these homologues can be classified into three types, long HPF, short HPF and YfiA.

Key words: hibernation promoting factor (HPF), 100S ribosome, ribosomal hibernation, stationary phase, translation.

Abbreviations: HPF, hibernation promoting factor; RMF, ribosome modulation factor.

INTRODUCTION

A shift from the exponential phase to the stationary phase of growth in *Escherichia coli* is accompanied by changes in the expression of >100 genes (*1*, *2*). Cells can live for a prolonged period of time in stationary phase by acquiring an increased resistance to environmental stress. The composition and conformation of ribosomes also changes drastically in stationary phase. We analysed the ribosome binding proteins of stationary phase cells, using the radical-free and highly reducing method of two-dimensional polyacrylamide gel electrophoresis (RFHR 2-D PAGE) (*3*) and reported the up-regulation of several ribosome binding proteins, including ribosome modulation factor (RMF) (*4*, *5*), SRA (stationary-phase-induced ribosome-associated protein) (*6*, *7*), YfiA and YhbH (*8*).

In stationary phase cells of *E. coli*, most of the 70S ribosomes are converted to 100S ribosomes (*4*, *9*). RMF, a small basic protein, binds near the peptidyl transferase (PTase) centre (*10*, *11*) and dimerizes 70S ribosomes to form 100S ribosomes, which are translationally inactive (*12*). Thus, 100S ribosomes constitute an inactive stored form of ribosomes during stationary phase and may be essential for survival during stationary phase. This resting state of the ribosome has been termed 'ribosomal hibernation' (*10*). The *rmf* deletion mutant and the Q13 strain, which cannot form 100S ribosomes, are unable to survive for prolonged periods in stationary phase (*9*, *13*). When stationary-phase cells are transferred to fresh medium, not only hibernation promoting factor (HPF) and YfiA, but also RMF rapidly dissociate from 100S ribosomes, which then become translationally active 70S ribosomes (*5*, *8*). This process is very quick, occurring within 1 min (*14*). The interconversion between active 70S and inactive 100S ribosomes may serve to regulate translational activity in response to environmental conditions (*10*).

Two small acidic proteins, HPF and YfiA, cannot be released from the ribosome by washing with high salt buffer, whereas RMF is released from 100S ribosomes. HPF and YfiA, however, can be released by low magnesium (1 mM Mg^{2+}) treatment, which dissociates 70S ribosomes into 30S and 50S subunits. These results have suggested that HPF and YfiA are located at the subunit interface (*8*).

Recently, the functions of HPF (YhbH) and YfiA in the *in vivo* formation of 70S dimers were examined using deletion mutants of HPF and YfiA (*15*). The *yfiA* deletion mutant expressed HPF and RMF in stationary phase and

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formed a greater number of 100S particles than the wild type, suggesting that HPF promotes and stabilizes 100S formation. In contrast, the *hpf* deletion mutant expressed YfiA and RMF, but produced no 70S dimers, suggesting that YfiA prevents 70S dimer formation. Thus, HPF and YfiA, a HPF paralogue, have opposite functions in 70S dimer formation despite sharing 40% sequence homology with each other. X-Ray analysis of the crystal structure of YfiA bound to the 30S subunit showed that YfiA binds to the P-site and A-site on the 30S subunit (*16*). Together with the high sequence homology between these two proteins, the amino acid similarity within the six amino acid residues of HPF and YfiA that contribute to ribosomal binding suggest that they interact with the same binding site and compete for binding to a region proximal to the P- and A-sites on 30S subunits. In the *hpf* and *yfiA* double deletion mutant, which expresses RMF, 70S dimers were observed as 90S particles. However, 100S particles were detected in an *yfiA* deletion mutant containing RMF and HPF. These results show that HPF probably converts immature 90S particles into 100S ribosomes (*15*). YhbH has been designated the 'hibernation promoting factor (HPF)' (*15*).

RMF homologues have been found only in the proteobacteria gamma group (*11*), but HPF homologues are found in almost all bacteria and also in plant plastids (*8*). What is the general role of HPF in translation? In this study, we analysed the functions of HPF in 100S ribosome formation *in vitro* to see if they would correlate with the ribosome behaviour previously observed *in vivo* in certain deletion mutants. Moreover, we examined the effects of HPF on *in vitro* protein synthesis. We report that HPF promotes the formation of 100S ribosomes *in vitro* as well as *in vivo*. It bound to ribosomes and inhibited incorporation in the poly (U)-dependent phenylalanine (phe) assay. In contrast, however, it neither bound to ribosomes nor inhibited incorporation in the MS2 RNA-dependent leucine (leu) assay. This suggests that bound HPF may function as a potential translational inhibitor. Together with the results of multiple alignment and phylogenetic analysis, our data provides insights into the general functions and characteristics of HPF as a hibernation promoting factor in bacterial species.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The *yfiA* and *hpf* double deletion mutant *E. coli* W3110 (Δ*yfiA* and Δ*hpf::Km*) (*15*) was used for *in vitro* binding and translation assays. The expression vector pQE-9 (QIAGEN, Hilden, Germeny), which contains the N-terminal 6x His-tag was used for cloning of the *hpf* gene. The ORF of *hpf* was amplified using W3110 genomic DNA as a template and the PCR primers 5′-gagcgaattcattaaagaggagaaattaactatgcagctcaacattaccg-3′ and 5′-cccaagctttttagtgttgtttcagtttatc-3′. We removed the 6x His-tag from the pQE-9 vector using these primers. The underlined nucleotides indicate the *EcoR*I and *Hind*III restriction sites, respectively. The amplified fragments were treated with the restriction enzymes *EcoR*I and *Hind*III and cloned into the *EcoR*I and *Hind*III sites of pQE-9 to generate the plasmid pQE-9-(Δ6xHis)-*hpf*. This plasmid was transformed into M15 pRep4 (QIAGEN) to generate M15 [pRep4, pQE-9-(Δ6xHis)-*hpf*]. The *E. coli* W3110 wild type strain was used for the preparation of the RMF protein. *Escherichia coli* Q13 was used for preparation of the S100 fraction for the *in vitro* translation system.

Growth Conditions—Each strain was grown in medium E containing 2% polypeptone and supplemented with 0.5% glucose (EP medium) at 37°C (*5*). When appropriate, kanamycin (25 μg/ml) and ampicillin (50 μg/ml) were added to the medium. W3110 (Δ*yfiA* and Δ*hpf::Km*) cells used for the preparation of ribosomes for both the *in vitro* binding assay and the *in vitro* translation assay were harvested after 2 days in stationary phase. W3110 cells used for the preparation of RMF protein were harvested after 4 days in stationary phase. Q13 cells for preparation of S100 and crude initiation factors were harvested at $OD_{600\,nm}$ 0.5 in exponential phase. Each cell pellet was stored at −80°C until use.

Preparation of High-salt Washed Ribosomes—Cells of W3110 (Δ*yfiA* and Δ*hpf::Km*) were harvested after 2 days in culture. The cell pellet was ground with an approximately equal volume of quartz sand (Wako, Osaka, Japan) and then extracted with association buffer (20 mM Tris–HCl [pH 7.6], 10 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol). Crude ribosomal fractions and high-salt washed ribosomes were prepared from the cell extracts essentially according to the method of Noll *et al*. (*17*) with slight modifications, as described (*18*).

Preparation of RMF—Crude ribosomes were prepared from *E. coli* W3110 cells after 4 days in stationary phase and resuspended in Buffer II (20 mM Tris–HCl [pH 7.6], 10 mM magnesium acetate, 1 M ammonium acetate, and 6 mM 2-mercaptoethanol). After 1 h at 4°C, the high-salt washed supernatant fraction was separated from ribosomes by centrifugation in a 55.2 Ti rotor (Beckman, Fullerton, CA, USA) for 4 h at 206,000*g*. The high-salt washing procedure was repeated once. The two high-salt supernatant fractions were combined and passed through Centriprep YM-10 and YM-3 (Millipore, Billerica, MA, USA) filters. Since the molecular weight of RMF is 6.476 kDa, RMF was recovered in the YM-10 ~ YM-3 fraction. After dialysis against association buffer and concentration (*12*), RMF protein was stored at −80°C until use.

Preparation of HPF—M15 pRep4 pQE-9-(Δ6xHis)-*hpf* cells were grown in EP medium at 37°C to OD_{600nm} 0.6. Expression was induced by the addition of 0.1 mM IPTG (isopropylthiol-β-D-galactoside). After an additional 1 h incubation, the cells were harvested and stored at −80°C. The cell pellets were thawed, ground with an approximately equal volume of quartz sand (Wako) and then extracted with association buffer. The cell debris was removed by centrifugation for 15 min at 14,000*g*. The ribosomes were removed by ultracentrifugation in a 55.2 Ti rotor (Beckman) for 2 h at 206,000*g* at 4°C. The supernatant was loaded onto a DE 52 column (Diethylaminoethyl cellulose; Whatman, Kent, UK) equilibrated with association buffer. As HPF proteins did not

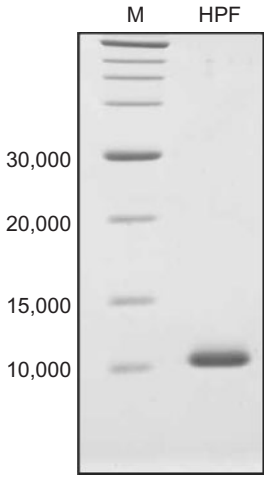

Fig. 1. **Purity of the purified HPF protein.** The purified fractions of HPF proteins were analysed by SDS-PAGE and CBB staining. HPF, 1.63 μg; molecular weight markers (10,000; 15,000; 20,000; 40,000; 50,000), 0.15 μg; molecular weight marker (30,000), 0.6 μg.

bind to the column, we collected the flow-through fraction and dialysed the fraction against 20 mM CH_3COOK (pH 5.0) buffer. The dialysed sample was loaded onto a CM 52 column (Carboxymethyl cellulose; Whatman) equilibrated with 20 mM CH_3COOK (pH 5.0) buffer. HPF was eluted with 140–200 mM LiCl in 20 mM CH_3COOK (pH 5.0). The fractions were combined, dialysed against 20 mM Tris-HCl (pH 7.6), 5 mM 2-mercaptoethanol and concentrated using an Amicon 5 K filter (Millipore). HPF protein was stored at −20°C in 50% Glycerol. The purified HPF protein was >90% pure as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

Analysis of Ribosome Dimers—HPF or RMF, or both proteins, were mixed with high-salt-washed 70S ribosomes at a molar ratio of 10:1 (protein/ribosome). After incubation at 37°C for 30 min, each mixture was layered onto a 5–20% sucrose density gradient in association buffer. The samples were then subjected to centrifugation in an SW40Ti rotor (Beckman) at 25,000*g* for 20 h at 4°C. The absorbance of each fraction was measured at 260 nm using a flow cell UV-1700 spectrometer (Shimadzu, Kyoto, Japan). The two units (absorbance 260 nm) of ribosome were used in all cases.

Western Blotting—After sucrose density gradient centrifugation, the samples were fractionated into 20 fractions. Proteins in each fraction were precipitated with 10% trichloroacetic acid (TCA), separated by 16% tricine SDS-PAGE (*19*) and transferred to PVDF membranes (Immobilon-FL Transfer membrane, Millipore) using a semidry transfer apparatus (Nihon Eido, Tokyo, Japan). HPF proteins and ribosomal protein L2 were detected with anti-HPF and anti-L2 antibodies. Alkaline phosphatase-goat anti-rabbit IgG (H+L) (Zymed Laboratories Inc., San Francisco, USA) was used as a secondary antibody and detected by employing the ECF substrate (GE Healthcare, Buckingham, UK) with a FLA 2000 imager (Fujifilm, Tokyo, Japan).

In vitro *translation assays*—S100 fractions and crude initiation factors were prepared from exponentially growing *E. coli* Q13 cells. *In vitro* translations were performed according to the method of Wada *et al*. (*12*), with slight modifications. Various amounts of HPF were mixed with the high-salt-washed ribosomes of the double deletions mutant and pre-incubated for 10 min at 37°C. Poly (U)-dependent phe incorporation was carried out for 30 min at 37°C in a 50 μl reaction mixture, containing 6 pmol ribosomes, 32 μg S100, 15 μg poly (U) (GE Healthcare), 15 μg tRNA (Sigma, St. Louis, MO, USA), 10 μM phenylalanine, 2 μM L-[*U*-^{14}C] phenylalanine (GE Healthcare; 16600 MBq/mmol), 53 mM Tris–HCl (pH 7.6), 60 mM ammonium chloride, 12 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 5 mM phosphoenolpyruvate, 1.5 μg phosphoenolpyruvate kinase and 6 mM 2-mercaptoethanol. After incubation of the samples, TCA was added and TCA-insoluble material was collected by filtration on a GF/C filter (Whatman), washed with cold 5% TCA and dried. Radioactivity on the filter was measured using a liquid scintillation analyser (2200CA, Packard Instrument Co., Inc., USA). For MS2 RNA-dependent leu incorporation, poly (U), phenylalanine and L-[*U*-^{14}C] phenylalanine were omitted and 8 μg MS2 RNA, 50 μM 19 amino acids (except leucine), 11.8 μg crude initiation factors containing IF1, IF2 and IF3 and 6.7 μM L-[*U*-^{14}C] leucine (GE Healthcare; 11000 MBq/mmol) were added, and the Mg^{2+} concentration was decreased to 8 mM. HPF binding to ribosomes in the *in vitro* translation system was analysed by western blot analysis. Poly (U) or MS2 RNA dependent *in vitro* translation was carried out using 30 pmol ribosomes without L-[*U*-^{14}C] phenylalanine or L-[*U*-^{14}C] leucine, and the reaction mixture was layered on a 5–20% sucrose density gradient in association buffer and then centrifuged in an SW40Ti rotor (Beckman) at 285,000*g* for 90 min at 4°C. The sample was fractionated into 18 fractions and the absorbance was measured at 260 nm using a UV-1700 spectrometer flow cell. Each fraction was precipitated by TCA and analysed by Tricine SDS-PAGE and western blotting using anti-L2 and anti-HPF antibodies.

Database Search and Phylogenetic Analysis—We used the FASTA search algorithm to examine the genomes of 12 eukaryotes, 30 bacteria and 9 archaea whose genome projects had been completed. The HPF and RMF homologues were downloaded from the KEGG database (*20*). The amino acid sequences of HPF homologues obtained by the FASTA search were aligned using the ClustalX program (*21*). The alignment was used for phylogenetic analysis using the PROTDIST and NEIGHBOUR programmes of the PHYLIP 3.6 package (*22*). The phylogenetic tree was inferred by the neighbour-joining method (*23*) and tested by 100 replications of the bootstrap analysis, which was carried out with the SEQBOOT and CONSENSE programs, and visualized using the TREEVIEW program (*24*).

RESULTS

Preparation of HPF and RMF—In order to examine HPF function *in vitro*, we purified HPF from M15

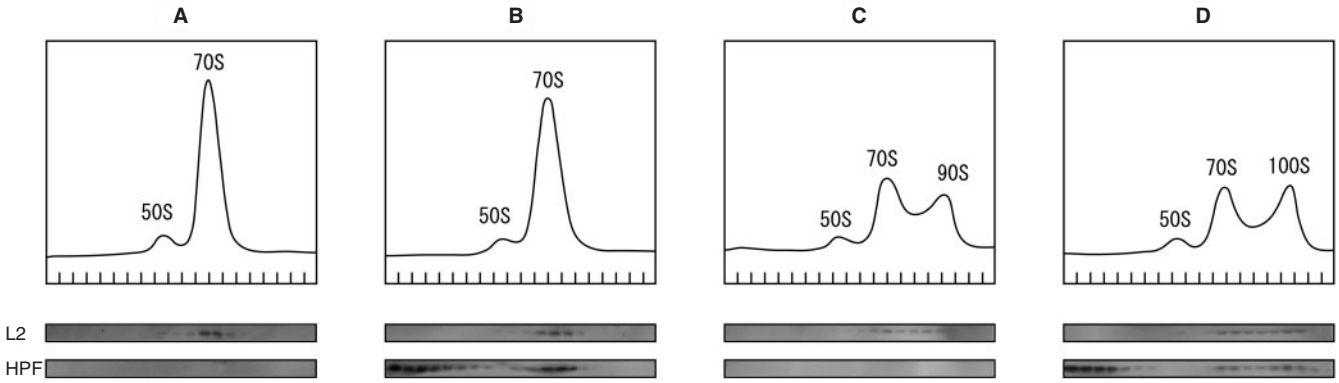

Fig. 2. ***In vitro* formation of 70S ribosome dimers.** High salt washed 70S ribosomes of W3110 (Δ*yfiA* and Δ*hpf::Km*) stationary phase cells were mixed with RMF or HPF, or both, layered onto a 5–20% sucrose density gradient and analysed by centrifugation. The upper panels show fractions of the 5–20% gradient, the left is the top of the gradient and the right is the bottom. *S*-values of 70S dimers were determined by normalizing the distance between the 50S and 70S particles. The experiments were performed at least five times. The lower panels show the western blot analysis using anti-L2 and anti-HPF. (A) ribosomes (B) ribosomes + HPF (C) ribosomes + RMF (D) ribosomes + HPF + RMF. In Fig. 2(D), we observed an increase (6%) in the amount of the 70S dimer and a decrease (6%) in the amount of the 70S monomer compared to those seen in Fig. 2(C).

(pRep4, pQE-9-(Δ6xHis)-*hpf*) cells induced to over-express HPF by IPTG treatment. Inhibition of cell growth by over-expression of HPF was not observed (data not shown). Purification of the HPF protein was performed as described in experimental procedures section. Ribosomes were removed and the supernatant was applied to a DE 52 column. The flow through fraction was collected, since HPF did not bind to the column. After dialysing, the flow through fraction was applied to a CM 52 column and eluted with a stepwise gradient of 10–200 mM LiCl in 20 mM CH_3COOK (pH 5.0). The HPF protein was eluted with 140–200 mM LiCl. SDS-PAGE of the purified HPF protein revealed a single band and the purity of HPF protein was >90% (Fig. 1). The RMF protein was purified using the method of Wada *et al*. (*12*). Purified HPF and RMF were used to examine the effect of HPF on *in vitro* formation of 70S dimers and *in vitro* protein synthesis.

Effect of HPF on the In vitro *Formation of 70S Dimers*—In the present study, to confirm the function of HPF in the *in vitro* formation of 100S ribosomes, we used purified HPF and RMF, and ribosomes that did not contain either HPF, YfiA or RMF. In previous *in vitro* work, we prepared and purified 70S ribosomes from stationary phase cells of the wild type W3110 strain by high salt washing. These 70S ribosomes were converted to 100S particles by RMF (*12*). However, these 70S ribosomes could have contained HPF, because it is very difficult to eliminate HPF by high salt washing alone. Therefore, in the present experiments, crude ribosomes were prepared from stationary phase cells (2 days) of *E. coli* W3110 (double mutant: Δ*yfiA* and Δ*hpf::Km*) (Fig. 2A) by high salt washing. This resulted in the dissociation of 100S particles into 70S particles that were completely free of HPF and RMF (data not shown).

After sucrose density gradient centrifugation, we confirmed which fractions contained 70S ribosomes by western blot analysis with anti-L2 antibody. The 70S ribosomes did not contain HPF protein, as shown by western blot analysis with anti-HPF antibody (Fig. 2A). When HPF was added to the 70S ribosomes, as described in the EXPERIMENTAL PROCEDURES section, HPF bound to the 70S ribosomes (Fig. 2B), but 70S dimers were not formed (Fig. 2B). In contrast, when RMF was added, the 90S dimer was generated (Fig. 2C). Lastly, when both RMF and HPF were added, the amount of the 70S dimer increased relative to the amount seen with RMF alone, and the dimers had an *S*-value of 100S (not 90S) (Fig. 2D). HPF bound to both 70S and 100S ribosomes (Fig. 2D). Thus, HPF first assists in the maturation of 70S dimers, and then, secondly, promotes 100S formation. These *in vitro* results are clearly consistent with previous work *in vivo* (*15*).

Effect of HPF on In vitro *Translational Activity*—When poly (U) is used as an mRNA in the *in vitro* translation system, physiologically significant activity is only observed in the elongation step. In contrast, when MS2 phage RNA, which has an initiation codon and a Shine-Dalgarno sequence in each of its four genes, was used as an mRNA in the *in vitro* translation, physiological significant activity is observed in every step, including initiation, termination and ribosome recycling, as well as the elongation step of translation (*12*, *25*). In contrast to the poly (U) system, translation in the MS2 RNA system is initiated by the normal initiation process (*26*) and requires initiation factors.

In this study, we examined whether HPF affects *in vitro* protein synthesis. We used HPF, RMF and 70S ribosomes that were prepared according to the same procedure as used in the *in vitro* assay of 100S ribosome formation. After the ribosomes were mixed with various amounts of HPF, they were subjected to *in vitro* translation in the poly (U) RNA system or the phage MS2 RNA system. The levels of poly (U)-dependent phe incorporation decreased depending on the amount of HPF (Fig. 3A). At a molar ratio of 5:1 (HPF:70S ribosome), inhibition by HPF was ~60%. At a molar ratio of 20:1, the inhibition was >80%. In addition, we investigated

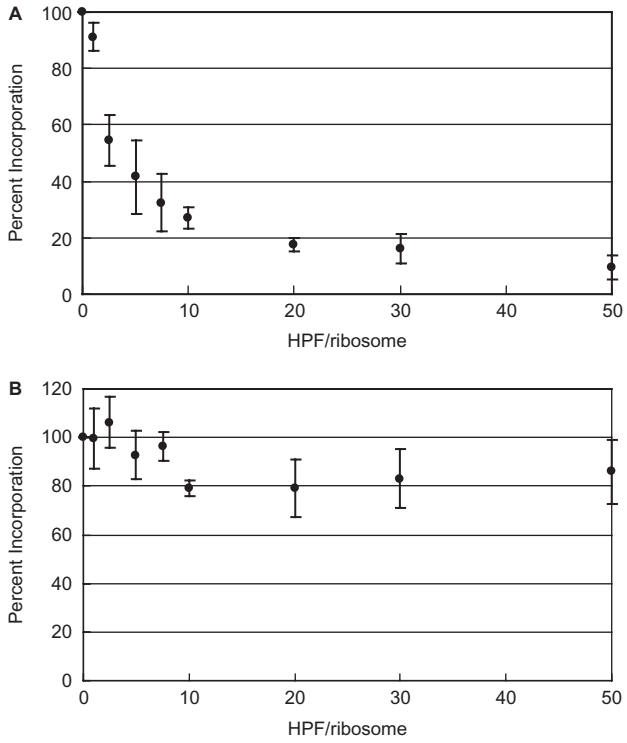

Fig. 3. **Effect of HPF on translational activity.** (A) poly(U)-dependent phe incorporation *in vitro* and (B) MS2-dependent leu incorporation *in vitro* were carried out in the presence of HPF proteins, as described in the EXPERIMENTAL PROCEDURES section. The level of protein synthesis was normalized to the level of no HPF addition. The value of 100% incorporation of phe (A) and leu (B) means 15.6 nmol and 1.7 nmol, respectively. The data shown are the average values of four independent experiments.

whether HPF binds to ribosomes in the poly (U) RNA system using western blot analysis, as described in EXPERIMENTAL PROCEDURES section (Fig. 4A). Bound HPF was retained on the ribosomes throughout the assay time, suggesting that bound HPF caused the inhibition. In contrast, in the phage MS2 RNA system, western blot analysis indicated that HPF was hardly bound to 70S ribosomes (Fig. 4B), and inhibition by HPF (at most 20%) was minimal (Fig. 3B). These data show that HPF probably neither inhibits initiation nor elongation in the MS2 RNA system. Additionally, the results indicated that bound HPF was removed from ribosomal particles during the assay probably by initiation factors (Figs 3B and 4B).

Phylogenetic Analysis of HPF, YfiA and RMF—HPF, YfiA and RMF are expressed in the stationary phase (*4*, *8*), and cooperatively control 100S ribosome formation (*8*, *15*). HPF homologues are known to exist in most bacteria (*8*, *27–29*) and even in plant plastids (*30–32*). Our homology search also confirmed that HPF is present in almost all bacteria and plant plastids. In contrast, RMF is present only in the proteobacteria gamma group (Table 1), suggesting that cooperative control of 100S ribosome formation by these three proteins developed in the proteobacteria gamma group.

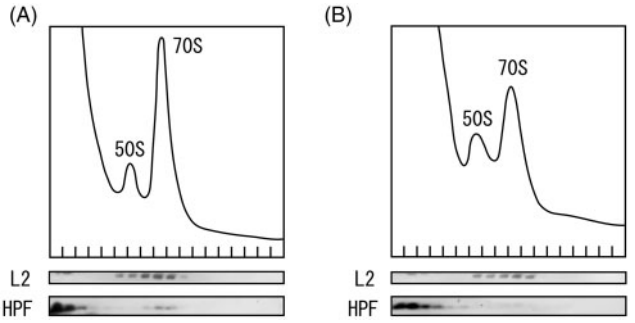

Fig. 4. **HPF binding to 70S ribosomes in the *in vitro* translation system.** (A) poly(U)-dependent phe incorporation *in vitro* and (B) MS2 RNA-dependent leu incorporation *in vitro* were carried out in the presence of HPF proteins and analysed by sucrose density gradient centrifugation and western blotting, as described in the EXPERIMENTAL PROCEDURES section. Upper panels: the fractions of the 5–20% gradient are shown with the top fractions at the left. The lower panels show the western blot analysis using anti-L2 and anti-HPF.

Multiple alignment and phylogenetic analysis of HPF homologues showed that HPF homologues can be classified into three types, long HPF, short HPF and YfiA (Figs 5A, B and Supplementary Data 1). All the homologues possess a conserved region (1–95 amino acids) near the N-terminus (Fig. 5A). Long HPF has a long tail region at the C-terminus and exists in most bacteria other than gamma and beta proteobacteria, whereas short HPF, which does not have the C-terminus tail region, exists in the gamma and beta proteobacteria. YfiA possesses a short region close to the C-terminus, and is present in gamma proteobacteria. Bacteria carrying long HPF do not have short HPF or YfiA (Table 1). Since the long *hpf* gene is widely conserved in bacteria and in plant plastids, long HPF is likely to be an ancestor of the HPF homologue. After a common ancestor of proteobacteria gamma and beta diverged from other bacterial species, long HPF appears to have lost the C-terminal tail region. Together with the fact that the YfiA specific region at the C-terminus is not similar to the C-terminal regions of long HPF, the acquisition of the YfiA specific region may have occurred after short HPF diverged from long HPF. Moreover, many proteobacteria gamma (including *E. coli*) have both short HPF and YfiA homologues, as well as RMF (Table 1). An exception, *Pseudomonas fluorescens*, has short HPF and RMF but not YfiA. *Xylella fastidiosa*, *Bordetella parapertussis and Nitrosomonas europaea* lack YfiA and RMF, and have only short HPF, which contains the conserved 1–95 amino acids and also a short C-terminal tail unlike YfiA. *Haemophilus infuluenzae* and *Pasteurella multocida* have lost the *hpf* gene and have only the YfiA homologue. This data suggest that changes in the C-terminal regions of HPF homologues play a critical role in controlling 100S ribosome formation by RMF.

DISCUSSION

In this study, we demonstrated that although HPF did not modify 70S ribosomes, RMF induced 70S ribosomes

Table 1. **The distribution of HPF, YfiA and RMF genes. The presence of the proteins is indicated by a +. An asterisk indicates that the homologous proteins were not found among annotated genes and proteins, but were found in genomic sequences. The short HPF homologue in *Yersinia pestis* contains a stop codon in the ORF.**

			HPF		RMF
		YfiA	short HPF	long HPF	
Bacteria					
Proteobacteria					
Gamma (enterobacteira)	*Escherichia coli W3110*	+	+		+
	Salmonella typhimurium	+	+		+
	Yersinia pestis	+	+*		+
	Shigella flexneri	+	+		+
	Erwinia carotovora	+	+		+*
	Klebsiella pneumoniae	+	+		+
	Serratia marcescens	+*	+*		+*
	Buchnera				
Gamma (others)	*Haemophilus influenzae*	+			
	Xylella fastidiosa		+		
	Vibrio cholerae	+	+		+
	Pseudomonas fluorescens		+		+
	Pasteurella multocida	+			
Beta	*Bordetella parapertussis*		+		
	Nitrosomonas europaea		+		
Epslion	*Helicobacter pylori*				
Delta	*Desulfovivrio desulfuricans*			+	
Alpha	*Rikettsia prowazekii*			+	
	Bradyhizobium japonicum			+	
Firmicutes					
Bacillales	*Bacillus subtilis*			+	
	Stapylococcus aureus			+	
Lactobacillales	*Streptococcus pyogenes*			+	
Clostridia	*Clostridium acetobutylicum*			+	
Mollicutes	*Mycoplasma genitalium*				
Actinobacteria	*Mycobacterium Tuberculosis*			+	
Chlamydia	*Chlamydia muridarum*				
Spirochete	*Borrelia burgdorferi*			+	
Cyanobacteria	*Thermosynechococcus elongatus*			+	
Green sulfur bacteria	*Deinococcus radiodurans*			+	
Hyperthermophilic bacteria	*Thermotoga maritimar*			+	
Eukaryotes					
Plants					
Dicotyledon	*Arabidopsis thaliana*			+	
	Oryza sativa japonica			+	
Red algae	*Cyanidoschyzon merolae*			+	

to form 90S dimers. Moreover, simultaneous addition of HPF and RMF converted the 90S particles to 100S ribosomes, and, furthermore, HPF increased the amount of 70S dimers. These *in vitro* results (Fig. 2) are consistent with those previously obtained *in vivo* (*15*).

In addition, we investigated the effects of HPF in *in vitro* translational assay systems. In poly (U)-dependent phe incorporation, HPF bound to ribosomes and inhibited translation (Fig. 3A). In contrast, in MS2 RNA-dependent leu incorporation, HPF hardly bound to ribosomes, and hardly inhibited translation (Fig. 3B). These two assay systems differ in their initiation processes; an artificial initiation process may be initiated by random binding of phe-tRNA to the P-site in the poly (U) system whereas a natural process is initiated in the MS2 system, which requires an SD sequence and an initiation codon in the mRNA, as well as initiation factors and f-met tRNA. In contrast, the elongation processes are the same in both assay systems. On the other hand, in our previous work, RMF inhibited the binding of N-formylmethionyl-tRNA to ribosomes in the MS2-RNA system and the binding of phe-tRNA in the poly (U) system (*12*). Thus, RMF inhibited protein synthesis in both systems. Taken together, these results

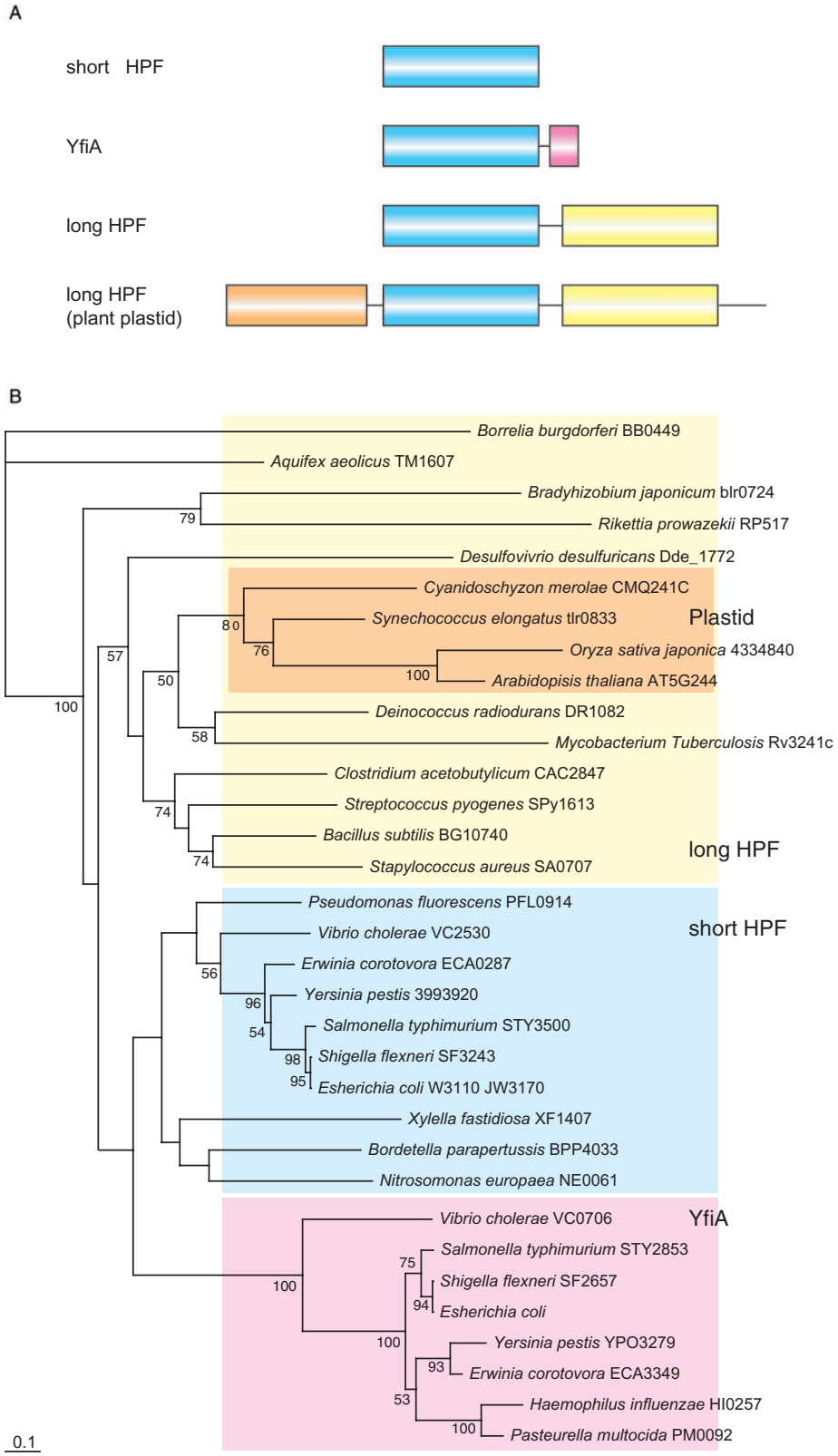

Fig. 5. **Comparison and phylogenetic analysis of HPF homologues.** (A) Three types of HPF homologues, YfiA, short HPF and long HPF were classified by the multiple alignment of HPF homologues. The long HPF of plant plastids has an additional sequence at the N-terminus. (B) Phylogenetic tree of HPF homologues. The short HPF, long HPF and YfiA branches of the trees are represented in blue, yellow and pink, respectively. The branches containing plant plastids and cyanobacterial derived genes are indicated in orange.

indicate that RMF is a stationary phase-specific inhibitor of ribosome function. The fact that RMF binds near the PTase centre (*11*) is consistent with the inhibition of protein synthesis by RMF.

Our results showing that HPF does not inhibit MS2 RNA-dependent leu incorporation may be because HPF is released from the subunit interface (Fig. 4B) during 70S ribosomal dissociation into 30S and 50S subunits by IF3 and IF1. Subsequently, f-Met-tRNA IF2 and GTP, and mRNA bind to the 30S ribosome. The f-met-tRNA occupies the P-site in the 30S initiation complex and 70S initiation complex (*26*). Therefore, HPF should neither be able to bind to the 30S subunit during normal initiation nor be able to inhibit initiation. HPF probably does not inhibit elongation in the MS2 RNA system. Maki *et al*. (*8*) also reported that YfiA and HPF (YhbH) were not present in the polysome fraction, indicating that HPF is released from ribosomes and cannot inhibit translation in living cells. HPF inhibition of translation in the poly (U) system may be because HPF is not released from the subunit interface of 70S ribosomes, since no initiation factors are present in this system (Fig. 4A). Thus, bound HPF must inhibit translation either by interfering with poly (U) entering the ribosome, or by preventing phe-tRNA binding to the P- or A-site.

An X-ray analysis of the crystal structure of YfiA suggests how HPF inhibits the binding of phe tRNA in the poly (U) system. The paralogous genes YfiA and HPF share 40% amino acid sequence homology. Vila-Sanjurjo *et al*. (*16*) reported that YfiA binds to both the P- and A-site on the ribosome by X-ray analysis of crystal structures. Of the six amino acids (Arg22, Lys25, Lys28, Lys79, Arg82 and Lys86) that are involved in ribosome binding by YfiA (*16*), four are conserved in HPF (Lys25, Lys79, Arg82 and Lys86) and one position has a Lys–Arg substitution (Lys22). Therefore, HPF may also bind to a ribosomal subunit interface near the A- and P-site (*15*), and inhibit phe-tRNA binding to the A-site and/or P-site.

In the stationary phase of growth, the translational activity of *E. coli* cells is lower than in the exponential growth phase, and most 70S ribosomes are converted to 100S ribosomes. HPF and RMF bind to 100S ribosomes. The translational activity of ribosomes prepared in the stationary phase is repressed by RMF for both poly (U)-dependent phe incorporation and MS2 RNA-dependent leu incorporation (*12*). RMF is a strong translational inhibitor, but HPF also binds selectively to 100S ribosomes. Inhibition of the translation in the poly (U) system suggests that ribosomal bound HPF may be able to inhibit translational activity of 100S ribosomes in living cells. In contrast, free HPF may not be able to bind to ribosomes and inhibit translation in translationally active cells. These results show that the hibernation system in *E. coli* involves reversible 100S ribosome formation and reversible translational inhibition, both of which are controlled by cooperation between RMF and HPF.

Multiple alignment and phylogenetic analysis of HPF homologues (Figs 5A, B) showed that HPF homologues could be classified into three types, long HPF, short HPF and YfiA. Most bacteria have at least one HPF homologue in their genomes, but animals, protists, fungi and archaea do not (data not shown). In contrast, RMF homologues are found in only the proteobacteria gamma group. The 100S ribosome hibernation system mediated by short HPF, YfiA and RMF may have evolved specifically in proteobacteria gamma.

We have identified 100S ribosomes and RMF proteins in enterobacteria such as *Salmonella typhimurium*, *Serratia marscescens* and *Proteus mirabilis* (*5*). We also observed 100S ribosome formation in enterobacteria, *Erwinia carotovora* and *Klebsiella pneumoniae* (data not shown). By database searching using the FASTA program, we found the *rmf* gene in the genomes of *E. carotovora* and *K. pneumoniae* (Table 1). The *rmf* gene of *E. carotovora* has not yet been annotated, but was identified by a homology search (Table 1). Both bacteria also have short HPF homologues. Other enterobacteria having RMF and short HPF homologues may also have 100S ribosome hibernation systems. As the six amino acids involved in ribosome binding are present in the N-terminal conserved region of HPF homologues, we predicted that HPF homologues may be able to bind to ribosomes. In fact, some HPF homologues have been reported to bind to ribosomes (*28*, *30–33*). We are investigating other organisms that have long HPF homologues, a short HPF, and YfiA but no RMF homologue, to see if they have 100S ribosomes or other hibernation systems. YfiA, a paralogue of HPF, was reported to inhibit translation at the elongation stage by blocking the binding of aminoacyl-tRNA to the ribosomal A-site (*34*). In *E. coli*, YfiA, a cold shock protein, inhibits translation initiation during cold shock but not at normal temperatures (*16*). Translational inhibition by YfiA, which stabilizes resting 70S ribosomes and prevents their dissociation, may be needed for the cold-shock-stress response in this system and may act in a different manner to inhibition by HPF (*35*, *36*).

In this study, we demonstrated that HPF promoted 100S ribosome formation *in vitro*, inhibited poly (U)-dependent phe incorporation, and hardly inhibited MS2 RNA-dependent leu incorporation. In the stationary phase growth in *E. coli*, most ribosomes were converted to 100S ribosomes, and lost their translational activity in the hibernation system. Although the hibernation system of the 100S ribosome mediated by HPF and RMF may exist only in proteobacteria gamma, HPF homologues exist in most bacteria. This suggests that other translational control systems mediated by HPF homologues may exist in many bacteria lacking RMF homologues.

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