Received: 16 February 2023 | Revised: 30 April 2024 | Accepted: 3 May 2024

DOI: 10.1111/gtc.13137

ORIGINAL ARTICLE

Genes to Cells WILEY

The hibernation promoting factor of Betaproteobacteria *Comamonas testosteroni* cannot induce 100S ribosome formation but stabilizes 70S ribosomal particles

Masami Ueta | **Akira Wada** | **Chieko Wada**

Biological Information Research, Yoshida Biological Laboratory Inc., Yoshida Biological Laboratory, Kyoto, Japan

Correspondence
Chieko Wada, Yoshida Biological Laboratory, 11-1 Takehanasotoda-cho, Yamashina-ku, Kyoto 607-8081, Japan.
Email: cwada@yoshidabio.co.jp; chiewada6@gmail.com

Communicated by: Hiroji Aiba

Abstract

Bacteria use several means to survive under stress conditions such as nutrient depletion. One such response is the formation of hibernating 100S ribosomes, which are translationally inactive 70S dimers. In Gammaproteobacteria (Enterobacterales), 100S ribosome formation requires ribosome modulation factor (RMF) and short hibernation promoting factor (HPF), whereas it is mediated by only long HPF in the majority of bacteria. Here, we investigated the role of HPFs of *Comamonas testosteroni*, which belongs to the Betaproteobacteria with common ancestor to the Gammaproteobacteria. *C. testosteroni* has two genes of HPF homologs of differing length (*Ct*HPF-125 and *Ct*HPF-119). *Ct*HPF-125 was induced in the stationary phase, whereas *Ct*HPF-119 conserved in many other Betaproteobacteria was not expressed in the culture conditions used here. Unlike short HPF and RMF, and long HPF, *Ct*HPF-125 could not form 100S ribosome. We first constructed the deletion mutant of C*thpf*-125 gene. When the deletion mutant grows in the stationary phase, 70S particles were degraded faster than in the wild strain. *Ct*HPF-125 contributes to stabilizing the 70S ribosome. *Ct*HPF-125 and *Ct*HPF-119 both inhibited protein synthesis by transcription-translation in vitro. Our findings suggest that *Ct*HPF-125 binds to ribosome, and stabilizes 70S ribosomes, inhibits translation without forming 100S ribosomes and supports prolonging life.

KEYWORDS

100S ribosome, Betaproteobacteria, *Comamonas testosteroni*, *Ct*HPF, C*thpf*-125 deletion mutant strain, hibernation promoting factor, in vitro translation assay, RFHR 2D PAGE, ribosome, stress response

1 | INTRODUCTION

Living cells make a large number of proteins to maintain vital functions. As an essential structural component of protein synthesis, ribosomes translate mRNAs into proteins and are conserved widely from prokaryotes to eukaryotes. Bacteria use several means to survive in severe environments or stress conditions such as nutrient depletion. In previous studies, we identified a novel bacterial stress response that involves the formation of 100S ribosomes (Wada et al., 1990; Yoshida & Wada, 2014). These translationally inactive "hibernating ribosomes" are dimers of 70S ribosomes. When the stressor is removed from the environment, 100S ribosomes

dissociate rapidly to reform translationally active 70S ribosomes. Therefore, translational control in response to stress involves the interconversion of active 70S and inactive 100S ribosomes (Yoshida & Wada, 2014).

During stress conditions in *Escherichia coli*, ribosome modulation factor (RMF) and hibernation promoting factor (HPF) are expressed and 100S ribosomes are formed by binding of RMF and HPF to 70S ribosomes (Izutsu et al., 2001; Maki et al., 2000; Maki & Yoshida, 2021; Ueta et al., 2005; Wada, 1998; Yamagishi et al., 1993; Yoshida et al., 2002; Yoshida & Wada, 2014). When *E. coli* cells are transferred from nutrient-depleted medium (starvation state) to fresh medium, RMF and HPF are released from the 100S ribosome, and the dissociated 70S ribosomes are translationally active (Maki et al., 2000; Wada, 1998). This process occurs within 1 min after the transfer (Aiso et al., 2005; Wada et al., 1990), and cells start to proliferate within 6 min. The 100S ribosome represents a resting form, and the process of 100S ribosome formation has been named the "hibernation" stage (Yoshida et al., 2002).

During the stationary phase, 100S ribosomes are formed in many bacterial strains. Two types of 100S ribosomes formation, RMF type and long HPF type, have been identified (Ueta et al., 2013): the RMF type is found in the Gammaproteobacteria class (Enterobacterales) containing *E. coli* and involves RMF and short HPF as hibernation factors (Maki et al., 2000; Ueta et al., 2005; Wada et al., 1990), whereas the long HPF type involves long HPF only as hibernation factor, and 100S of this type is formed in the majority of other bacterial species, including *Staphylococcus aureus* (Basu & Yap, 2016; Ueta et al., 2010; Ueta et al., 2013), *Bacillus subtilis* (Tagami et al., 2012), *Lactococcus lactis* (Puri et al., 2014), *Lactobacillus paracasei* and *Thermus thermophilus* (Ueta et al., 2013), *Listeria monocytogenes* (Kline et al., 2015), and *Streptomyces venezuelae* (Jones et al., 2014). A structural analysis of the RMF type revealed that 100S ribosome dimerization involves a back-to-back interaction of the 30S subunits of each 70S monomer (Beckert et al., 2018; Kato et al., 2010; Ortiz et al., 2010; Polikanov et al., 2012). On the other hand, a cryoelectronic microscopy analysis showed that long HPF-induced 100S dimerization involves platform-to-platform interaction of 30S subunits via binding of the C-terminal tail regions of two long HPF molecules (Beckert et al., 2017; Flygaard et al., 2018; Franken et al., 2017; Khusainov et al., 2017; Matzov et al., 2017).

In *E. coli*, in addition to the hibernation factors HPFs and RMF, YfiA (RaiA) also induced by cold shock and in the stationary phase associates with 70S ribosomes and participates in the modulation of ribosome (Agafonov et al., 2001; Maki et al., 2000). YfiA protect ribosomes by protecting ribonuclease target sites within the ribosome (Agafonov et al., 1999; Prossliner et al., 2021). Binding of YfiA interferes with translation initiation (Vila-Sanjurjo et al., 2004) and elongation (Agafonov et al., 2001) by blocking mRNA-dependent tRNA binding in the P site and inhibiting tRNA binding to the A site (Vila-Sanjurjo et al., 2004). Furthermore, Since *Ec*HPF and YfiA have the same binding site, there is a competition for the binding site in normal strains, resulting in a higher 100S yield in YfiA deletion strains (Ueta et al., 2005).

The HPF homologs long HPF, short HPF, and YfiA (RaiA) are conserved widely in bacteria and plant plastids (Sharma et al., 2007; Ueta et al., 2008; Ueta et al., 2013). Genes encoding long HPF exist in the majority of bacteria, except Gammaproteobacteria and Betaproteobacteria. According to a phylogenetic tree of prokaryotes, the Alphaproteobacteria class and a common ancestor of Gammaproteobacteria and Betaproteobacteria classes diverged first, and then the Gammaproteobacteria and Betaproteobacteria classes diverged many years later (Battistuzzi & Hedges, 2009). During the first divergence of the Alphaproteobacteria class, which has a long HPF, the common ancestor appears to have lost about 50% of the C-terminal region of long HPF essential to 100S ribosomes formation. During the second divergence, the Gammaproteobacteria class diverged from the Betaproteobacteria class, and acquired the *rmf* gene. Consequently, for example, in *E. coli*, it was established a new different system to generate 100S ribosomes by RMF and short HPF (Figure S5). In the stationary phase of *E. coli*, RMF and short HPF expressed in large amounts. RMF binds to 70S ribosomes, and forms 90S ribosome. Next, short HPF binds to the 90S, and completes tight 100S (Ueta et al., 2005).

E. coli HPF (*Ec*HPF) lacks the C-terminal tail sequence required for dimerization of the 70S ribosome; as such, it can only induce formation of the 100S ribosome in the presence of *Ec*RMF. On the other hand, 100S ribosomes were not formed in the Betaproteobacteria species *Burkhordelia multivorans*, which lacks RMF and has a *Bm*HPF homolog lacking the C-terminal region (Ueta et al., 2013). To understand the hibernation mechanism in Betaproteobacteria in more detail, we analyzed the ribosomes of the Betaproteobacteria species *Comamonas testosteroni*, which has no *rmf* gene but two *hpf* genes that encodes HPF lacking the full C-terminal region of long HPF. *C. testosteroni* exists naturally in soil and water and has also been identified as a clinical specimen.

The *C. testosteroni* TK102 strain (NBRC 109938) was isolated from soil contaminated with polychlorinated biphenyls in Hamamatsu, Japan (Fukuda et al., 2014). Because it is resistant to heavy metals and can utilize biphenyl as a sole carbon source and degrades polychlorinated biphenyls, *C. testosteroni* is useful for waste treatment and bioremediation. Moreover, steroid degradation

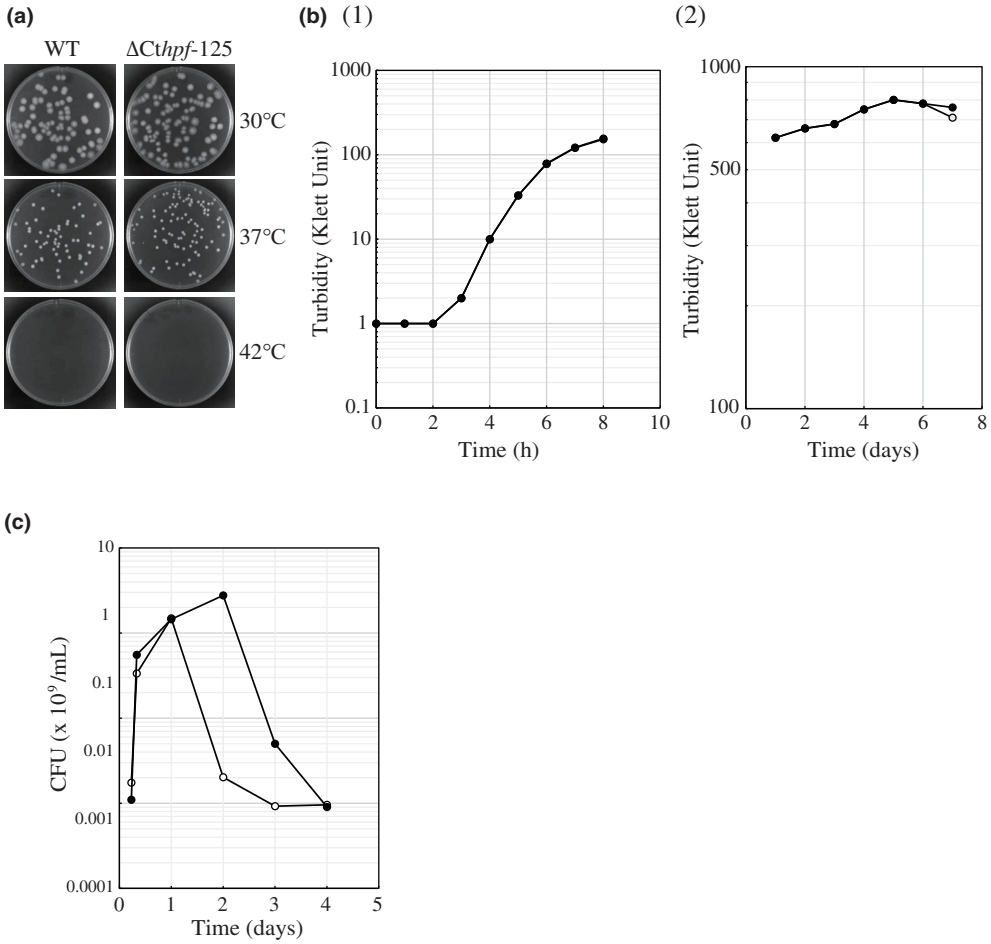


FIGURE 1 Growth of *C. testosteroni* ATCC11996 wild type and C*thpf*-125 mutant in liquid and solid 802-medium at various temperatures. (a) Bacterial colony sizes on solid 802-medium. *C. testosteroni* wild and C*thpf*-125 mutant cells were cultured overnight at 30°C in liquid 802-medium and then diluted and spread onto 802-medium plates. The plates were incubated at 30, 37, or 42°C for 2 days and the photographs were shown. (b) Growth curves of *C. testosteroni* wild and C*thpf*-125 mutant cells cultured in liquid 802-medium at 30°C. Cell growth was monitored via turbidity (Klett units). Panels (1) and (2) show different time scales (0–8 h and 1–7 days, respectively). The vertical axes show normal logarithmic values. Filled and open circles show the wild type and the C*thpf*-125 mutant cells, respectively. (c) Colony forming unit (CFU) values of *C. testosteroni* cultured wild and C*thpf*-125 mutant cells in liquid 802-medium at 30°C. Cultured cells were sampled every day for 4 days, and the CFU value was measured. The vertical axis shows normal logarithmic values CFU ($\times 10^9$)/mL. Filled and open circles show the wild type and the C*thpf*-125 mutant cells, respectively.

by *C. testosteroni* has also been described (Horinouchi et al., 2018).

Here, we used *C. testosteroni* ATCC11996 (NBRC 14951^T) (Tamaoka et al., 1987) and examined the fundamental functions of *Ct*HPF expressed under stress conditions by analyzing deletion mutant strains.

2 | RESULTS

2.1 | Betaproteobacteria *C. testosteroni* grows at 30°C

C. testosteroni ATCC11996 (NBRC 14951^T) wild type strain and the deletion mutant of C*thpf*-125 gene isolated from *C. testosteroni* wild type strain were cultured for overnight (16 h) at 30°C in liquid 802-medium, and then the cells were diluted and spread onto 802-medium agar plates. The plates were incubated in the incubator for 2 days at 30, 37, or 42°C, and were taken by photo. At each temperature, the size and number of colonies did not differ between the wild type and the mutant strain. The wild and mutant cells incubated at 30°C grew better than those incubated at the higher temperatures (Figure 1a); therefore, *C. testosteroni* was cultured at 30°C in subsequent experiments. When two *C. testosteroni* were cultured in liquid 802-medium at 30°C, the doubling time in the logarithmic phase was 31–34 min (Figure 1b[1]), and the mutant strain did not differ from the wild-type strain. In the stationary phase, cell turbidity

(Klett units) of the wild and the mutant strain was almost the same increase across a 6 days period but the mutant strain subsequently decreased on the 7th day unlike the wild strain. (Figure 1b[2]). However, the number of colony forming units of the wild type strain increased two generations for the first 2 days and decreased thereafter, but that of the deletion strain increased during 1 day and decreased thereafter (Figure 1c).

2.2 | The genome of *C. testosteroni* ATCC11996 encodes two HPF homologs

The genome of *C. testosteroni* encodes two HPF homologs of 125 and 119 amino acids (hereafter named *Ct*HPF-125 and *Ct*HPF-119, respectively). We performed an amino acid alignment analysis of *Ct*HPF-125, *Ct*HPF-119, *Ec*HPF (as an example of short HPF), *L. paracasei* HPF (*Lp*HPF; as an example of long HPF), and *Ec*YfiA (Figure 2). Although the C-terminus of HPF differs, its N-terminus is conserved in a large number of bacteria. *Ec*HPF and *Ec*YfiA have a β1α1β2β3β4α2 topology with disordered terminal residues (Rak et al., 2002; Sato et al., 2009; Ye et al., 2002). The structures of *Ct*HPF-125 and *Ct*HPF-119 predicted by amino acid sequence homology modeling are similar to those of *Ec*YfiA, *Ec*HPF, and *Lp*HPF, with two main exceptions. Specifically, *Ct*HPF-125 and *Ct*HPF-119 are 20 and 19 amino acids longer than *Ec*HPF at the C-terminal region, respectively, and both include additional amino acids in the 50–55 region

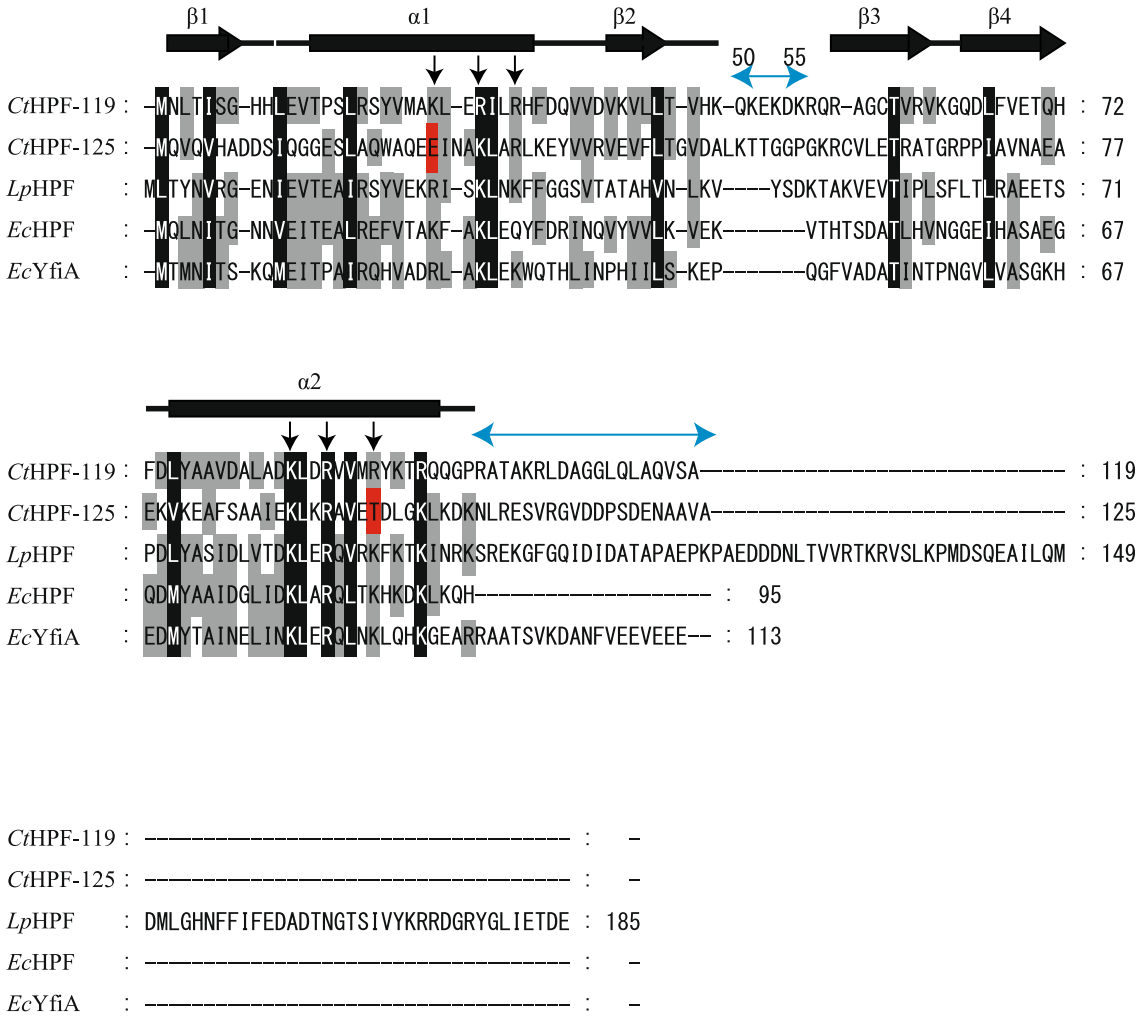


FIGURE 2 Amino acid sequence alignment of *Ct*HPF-119, *Ct*HPF-125, *Lp*HPF (long HPF), *Ec*HPF (short HPF), and *Ec*YfiA (paralog of *Ec*HPF). Black and gray shading indicate residues that are identical between five and four or three sequences, respectively. The overall structure of the HPF homologs is shown above the sequence by boxes (alpha helices) and arrows (beta sheets). The downward small arrows indicate the six amino acids involved in ribosome binding in *Ec*YfiA (Vila-Sanjurjo et al., 2004). Red shading indicates the first and sixth ribosome binding-associated amino acids, which differ in *Ct*HPF-125. The short blue arrow indicates the unique sequences of HPF homologs from Betaproteobacteria. The long blue arrow indicates the C-terminal extension, which does not exist in *Ec*HPF.

between the β2 and β3 sheets (Figure 2). The six amino acids of *Ec*YfiA involved in ribosome binding (Vila-Sanjurjo et al., 2004) are mostly conserved across the HPF proteins examined (downward arrows in Figure 2), although *Ct*HPF-125 differs in two of the six amino acids. Overall, approximately 24% of the amino acid residues of *Ct*HPF-125 and approximately 35% of those of *Ct*HPF-119 are identical to those of the corresponding regions of *Ec*HPF (Figure 2).

2.3 | Analysis of the ribosomal proteins of *C. testosteroni* ATCC11996

To analyze the ribosomal proteins (r-proteins) of *C. testosteroni* ATCC11996, high-salt washed ribosomes (HSRs) and both subunits (50S and 30S) fractions were prepared from logarithmic growth phase cells, as described in the Section 4. The r-proteins in the HSR, 50S, and 30S fractions were extracted using the acetic acid method and analyzed by radical-free and highly reducing 2D polyacrylamide gel electrophoresis (RFHR 2D PAGE). The position of each *C. testosteroni* r-protein on the RFHR 2D PAGE gel was predicted by comparing the theoretical molecular weight (MW) and isoelectric point (pI) computed from the amino acid sequence of each r-protein with the pattern of the *E. coli* r-proteins on an RFHR 2D PAGE gel (Wada, 1986a, 1986b). Some spots (L10, L17, L19, L22, L27, L33, S11, and S17) were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). All r-proteins of *C. testosteroni* ATCC11996 that existed in the 50S fraction (L1–L36, except L8 and L26) and the 30S fraction (S1–S21) were located as spots on the RFHR 2D PAGE gel (Figure 3).

Next, crude ribosome (CR) and HSR fractions were prepared from *C. testosteroni* cells in the logarithmic growth and stationary phases. We searched for candidate spots on the RFHR 2D PAGE gel representing *Ct*HPF-125 (125 amino acids; MW 13,513; pI 5.52) and *Ct*HPF-119 (119 amino acids; MW 13,524; pI 9.93). Assuming that these proteins promote bacterial hibernation, we expected the spots to be present only in the stationary phase samples. Using MALDI-TOF MS, *Ct*HPF-125 was identified as a spot located near to the L7/L12 r-protein, but a candidate spot representing *Ct*HPF-119 was not found (Figure 3b). High-salt washing of CRs (i.e., the HSR fraction) released *Ct*HPF-125 from the ribosome particles (Figure 3b). These findings suggest that *Ct*HPF-125 is expressed only in the stationary phase and bound to ribosomes. No *Ct*HPF-125 protein was identified in the RFHR electrophoresis patterns of the CR and post ribosomal supernatant (PRS) fractions of the *Cthpf*-125 deletion mutant strains even in the stationary phase (Figure S2).

2.4 | *Ct*HPF-125 existed in the CR and PRS fractions of stationary phase cells

To investigate how the expression of *Ct*HPF-125 changes with culture time, the CR fraction and the total protein, the latter of which comprises the CR, PRS, and cell debris (CD) fractions, were prepared from cells in the logarithmic growth phase for 5 h (Klett units = 50) and the stationary phase (10 h, 15 h, 1 day, and 2 days). RFHR 2D PAGE analysis of these samples identified *Ct*HPF-125 in the CR fraction and total protein of stationary phase cells only (Figure 4a[1,2]), suggesting that *Ct*HPF-125 is bound to ribosomes in the CR fraction of the cells.

Next, the densities of the *Ct*HPF-125 spot and the r-protein spots in the *Ct*HPF-125-adjacent region on the RFHR 2D PAGE gel were measured using a densitometer, and the copy number of each protein was calculated. The copy number of *Ct*HPF-125 in the CR fraction increased during the stationary phase (10 and 15 h), reached a maximum at 1 day, and thereafter decreased (Figure 4a[1],b). Moreover, the copy number of *Ct*HPF-125 in the total protein fraction increased and decreased in a similar manner (Figure 4a[2],b); however, the copy number of *Ct*HPF-125 in the total protein fraction was larger than that in the CR fraction at each stationary phase time-point. The difference between the copy numbers of *Ct*HPF-125 in the CR fraction and that of the free form (total protein minus CR) became larger as the time in the stationary phase increased (Figure 4b). These findings suggested that only a subset of *Ct*HPF-125 proteins binds to ribosomes, and a larger subset exists in a free state in the PRS fraction. To test this hypothesis, PRS fractions were prepared from cells in the logarithmic phase (5 h) and the stationary phase (10 h, 15 h, 1 day, and 2 days), and the proteins were analyzed by RFHR 2D PAGE. *Ct*HPF-125 was found in all stationary phase PRS fractions and existed stably even after culture of the cells for 2 days (Figure 4a[3]), indicating that, in addition to binding to ribosomes, it can also exist stably in a free state.

The CR fraction is typically prepared by ultracentrifugation with a 30% sucrose cushion (Ueta et al., 2013). When the CR fraction of *C. testosteroni* cells cultured for 1 day was prepared with a 30% or 50% sucrose cushion, the copy number of *Ct*HPF-125 was 0.26 or 0.16, respectively, whereas it was 0.4 without a sucrose cushion. This finding suggests that *Ct*HPF-125 bound to ribosomes was released by ultracentrifugation with the sucrose cushion, and that the ability of *Ct*HPF-125 to bind to ribosomes is weaker than those of other HPF homologs such as *Ec*HPF and long HPF. Consequently, in subsequent experiments, we prepared CR fractions without the use of a sucrose cushion.

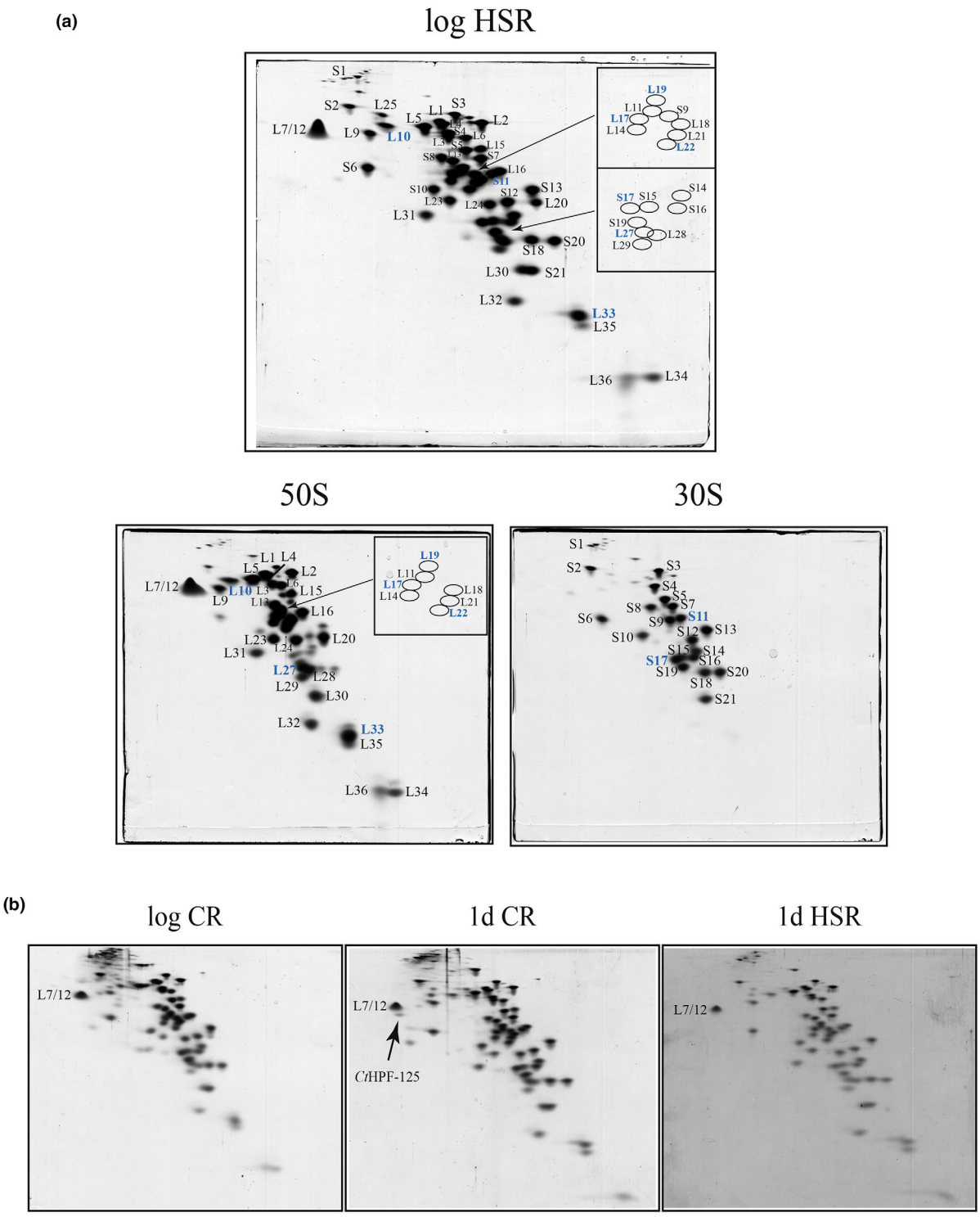


FIGURE 3 Proteome analysis of ribosomal fractions of *C. testosteroni* ATCC11996 using RFHR 2D PAGE. (a) The upper column shows the RFHR 2D PAGE pattern of HSRs prepared from *C. testosteroni* cells in the logarithmic phase (5 h). The lower column shows the RFHR 2D PAGE pattern of the 30S and 50S subunits prepared from the HSR sample. The spots shown as blue characters (L10, L17, L19, L22, L27, L33, S11, and S17) were identified by MALDI-TOF MS. The positions of the other proteins were estimated based on r-proteins belonging to the 30S or 50S subunit, MW, and pI. The position of each *E. coli* r-protein spot was used as a reference. (b) Identification of *Ct*HPF-125. CR samples were prepared from cells in the logarithmic phase (5 h), and CR and HSR samples were prepared from cells in the stationary phase (1 day). The arrows indicate the spots representing *Ct*HPF-125.

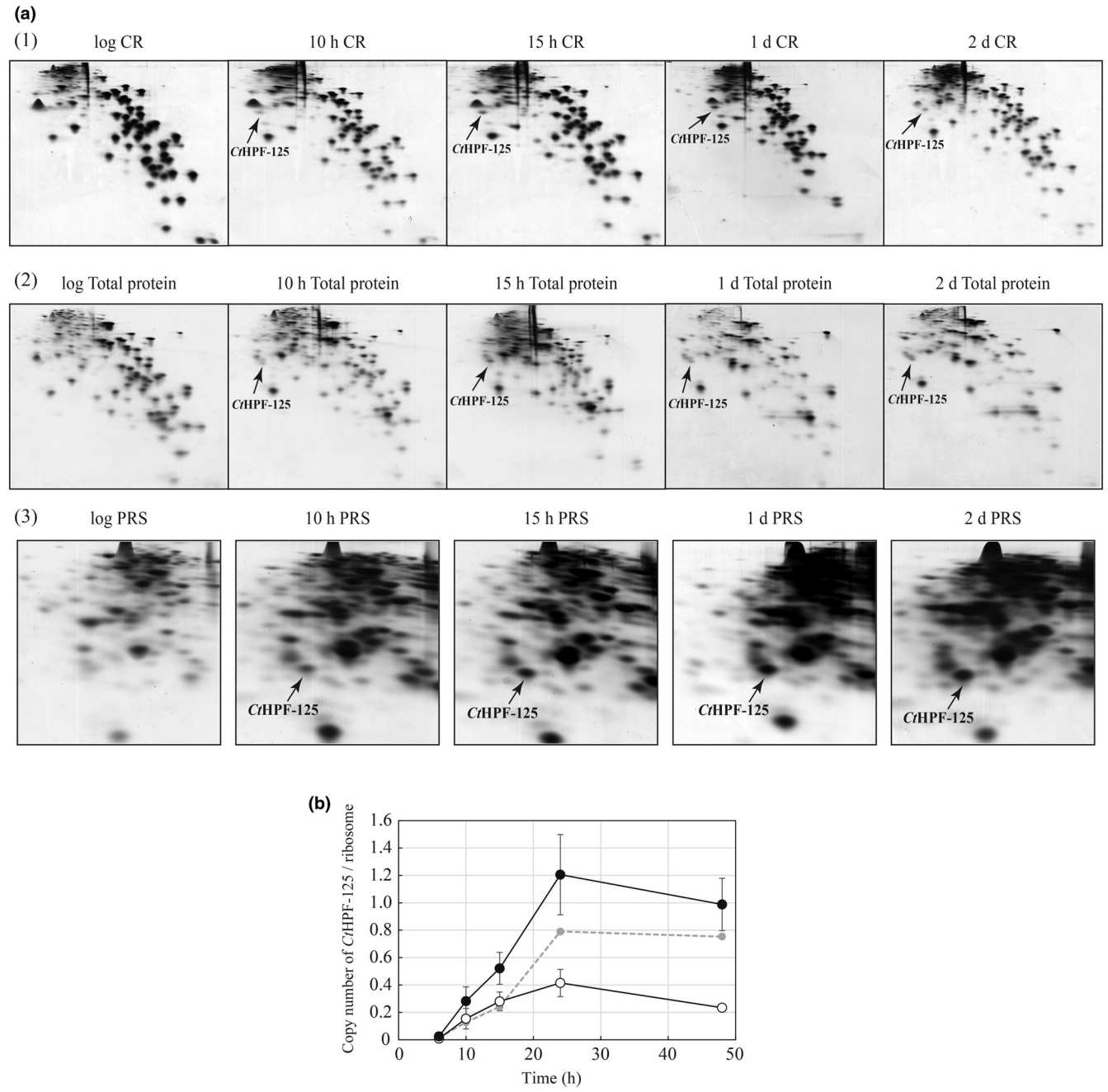


FIGURE 4 The expression of *Ct*HPF-125 in the CR, total protein and PRS fractions of cells in the logarithmic and stationary phases. (a) RFHR 2D PAGE patterns of the CR (1), total protein (2), and PRS (3) fractions of cells in the logarithmic phase (5 h) and stationary phase (10 h, 15 h, 1 day, and 2 days). The spots representing *Ct*HPF-125 are indicated by arrows. (b) The copy numbers of *Ct*HPF-125 in the CR (open circles) and total protein (filled circles) fractions of the cells described in (a). The gray broken line indicates the difference (CD + PRS fractions) between the copy numbers of *Ct*HPF-125 in the CR and total protein fractions. Data are represented as the mean ± SEM of at least five independent experiments.

2.5 | *Ct*HPF-119 is not expressed at any growth phase

Purified *Ct*HPF-119 synthesized in *E. coli* was able to bind to ribosomes of *C. testosteroni* (*Ct*-ribosomes) in vitro and was located close to the L7/L12 (L17) spot on an RFHR 2D PAGE gel (Figure 5a). However, as mentioned above, the RFHR 2D PAGE analysis did not detect native *Ct*HPF-119 in the CR fraction or total protein of logarithmic growing or stationary phase cells (Figure 4a). To detect potential low-level expression of *Ct*HPF-119, the cell extract (CE), CR, and PRS fractions of

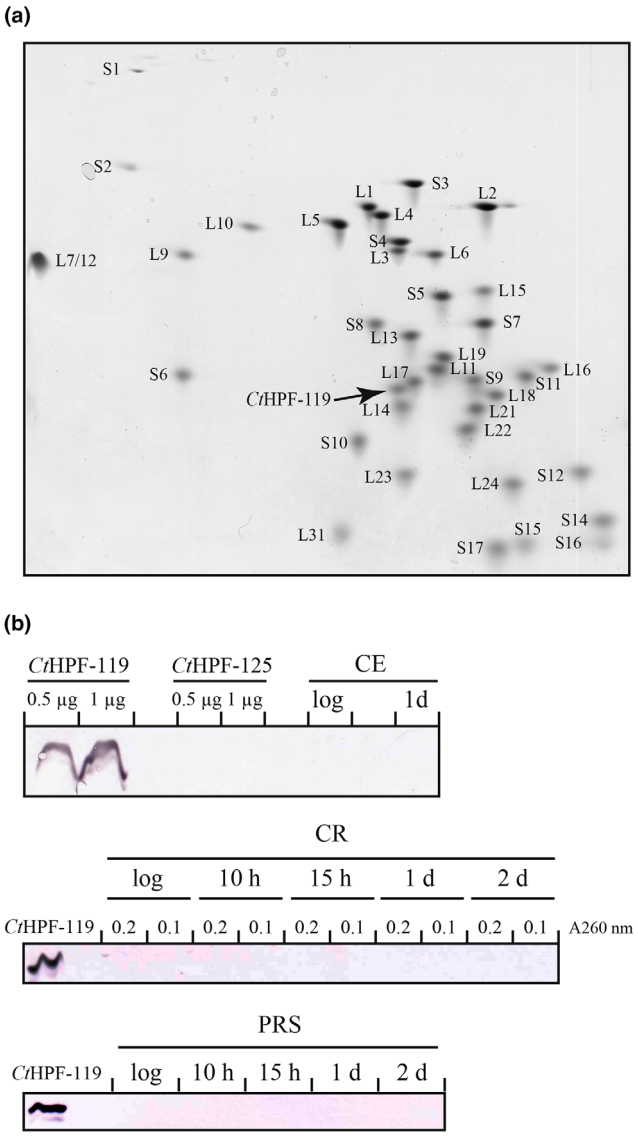


FIGURE 5 *Ct*HPF-119 binds to ribosomes in vitro but is not expressed in *C. testosteroni* cells during any growth phase. (a) HSRs were isolated from *C. testosteroni* cells in the logarithmic phase and incubated with purified *Ct*HPF-119 for 30 min at 37°C. The mixture was then centrifuged to collect the ribosomes, and r-proteins were analyzed by RFHR 2D PAGE. The arrow indicates the spot near L17 representing *Ct*HPF-119. (b) Western blot analysis using an anti-*Ct*HPF-119 antibody. CE fractions were prepared from *C. testosteroni* cells in the logarithmic phase (5 h; A260 nm = 0.5, A280 nm = 0.23) and stationary phase (1 day; A260 nm = 0.32, A280 nm = 0.15) (upper image). CR fractions (middle image; A260 nm = 0.1 or 0.2) and PRS fractions (lower image; 20 µL, A280 nm = 0.1–0.2) were also prepared from cells in the logarithmic phase (5 h) and stationary phase (10 h, 15 h, 1 day, and 2 days). The fractions were separated on 12% SDS-PAGE gels and analyzed by immunoblotting with an anti-*Ct*HPF-119 antibody. Purified *Ct*HPF-119 and *Ct*HPF-125 (0.5 µg or 1.0 µg) were loaded as controls in the upper image, and purified *Ct*HPF-119 (0.5 µg) was loaded as a control in the middle and lower images.

C. testosteroni were analyzed by western blotting using an antibody of *Ct*HPF-119. The anti-*Ct*HPF-119 antibody reacted with *Ct*HPF-119 but not with *Ct*HPF-125 (Figure 5b, upper image). No *Ct*HPF-119 immunoreactive bands were detected in the CE, CR, and PRS fractions of cells in the logarithmic growth and stationary phase (Figure 5b). These results indicate that *Ct*HPF-119 is not expressed at any growth phase in the culture conditions used here.

2.6 | *Ct*HPF-119 and *Ct*HPF-125 are not expressed during cold or heat stress

*Ec*YfiA, a homolog of *Ec*HPF, is induced by cold shock (Agafonov et al., 2001). Therefore, we examined whether *Ct*HPF-119 and *Ct*HPF-125 are expressed in cells during cold or heat shock. To this end, cells in the logarithmic growth phase were cultured at 20°C for 2 h (cold shock) or at 37°C for 1 h (heat shock). Subsequently, CR factions were prepared and analyzed by RFHR 2D PAGE. *Ct*HPF-119 and *Ct*HPF-125 were not detected in the CR fraction of either cell type (Figure 6a). In addition, western blotting of the CR and PRS fractions of cells cultured under cold or heat shock conditions did not detect *Ct*HPF-119 immunoreactive bands (Figure 6b). These results suggest that *Ct*HPF-119 and *Ct*HPF-125 are not induced by cold or heat shock.

2.7 | Purified *Ct*HPF-119 and *Ct*HPF-125 bind to *C. testosteroni* ATCC11996 ribosomes in vitro

As *Ct*HPF-119 expression was not detected in vivo, we analyzed its function in vitro using a ribosome binding assay. The HSR fraction prepared from *C. testosteroni* cells cultured for 1 day was incubated with purified *Ct*HPF-119 (10-fold) and/or *Ct*HPF-125 (10-fold) at 30°C for 30 min. Subsequently, the mixtures were centrifuged with 30% sucrose bed to avoid contamination of free *Ct*HPF into the ribosomal fraction, and the ribosomes were collected. The r-proteins of the ribosomal fractions were then analyzed by RFHR 2D PAGE. The ribosome binding assay revealed that 0.47 copies of *Ct*HPF-119 and 0.27 copies of *Ct*HPF-125 were bound to each ribosome (Figure 7a). When *Ct*HPF-119 and *Ct*HPF-125 were added to the ribosome at same time, the copy number of bound *Ct*HPF-119 increased to 0.54 (0.07 copies higher than the single addition), whereas that of *Ct*HPF-125 was reduced to 0.20 (0.07 copies lower than the single addition; Figure 7a).

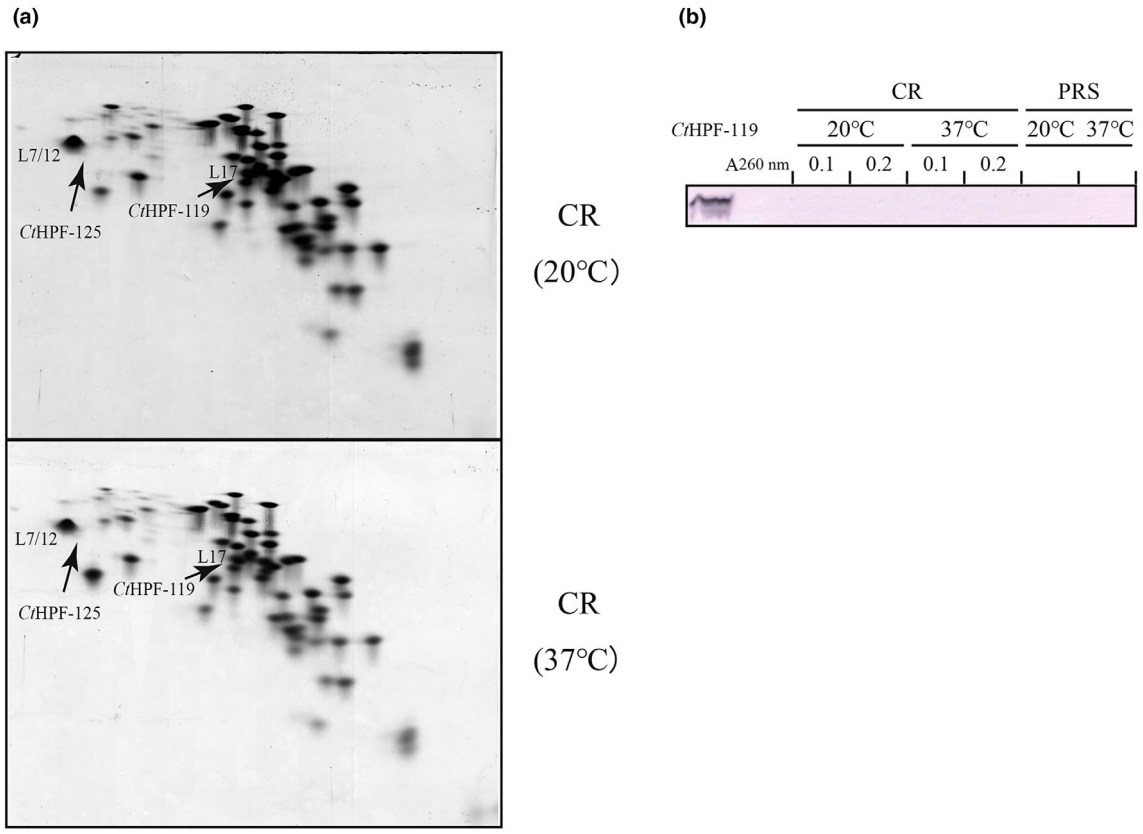


FIGURE 6 *Ct*HPF-119 and *Ct*HPF-125 are not expressed at low (20°C) and high (37°C) temperatures. (a) CR fractions were prepared from cells cultured in cold or heat shock conditions and were analyzed by RFHR 2D PAGE. (b) Western blot analysis of *Ct*HPF-119 in CR (A260 nm = 0.1 or 0.2) and PRS (20 μL; A_{280} 0.09 at 20°C or A_{280} 0.15 at 37°C) fractions prepared from cells cultured in cold or heat shock conditions. Purified *Ct*HPF-119 (0.5 μg) was loaded as a control.

2.8 | Purified *Ct*HPF-119 and *Ct*HPF-125 inhibit translation activity in vitro

Next, we used an in vitro transcription-translation PURE assay system (Shimizu et al., 2005) to investigate whether *Ct*HPF-119 and *Ct*HPF-125 can affect translational activity. *Ct*-ribosomes were prepared from *C. testosteroni* cells in the logarithmic growth and stationary phases. As DNA template, the gene encoding modified *E. coli* glutathione S-transferase (*Ec*GST; 201 amino acids), harboring a deletion (194–201) of C-terminal amino acids 1–193 was used to distinguish the synthesized protein band from the bands of *C. testosteroni* r-proteins and other proteins contained in the reaction mixture. The addition of *Ct*HPF-125 or *Ct*HPF-119 to the in vitro transcription-translation assay mixture reduced translational activity in a dose-dependent manner (Figure 7b[1]). For both proteins, the inhibitory effects were similar on ribosomes extracted from cells in the logarithmic and stationary growth phases; however, the inhibitory effect of *Ct*HPF-119 was much more pronounced than that of *Ct*HPF-125 (Figure 7b[1]), which may be a result of its stronger ribosome binding activity (Figure 7a). When a 10:1 molar ratio of *Ct*HPF-125 or *Ct*HPF-119 to ribosome was added to a reaction mixture containing *Ct*-ribosomes prepared from cells cultured for 1 day, translation was inhibited by approximately 18% by *Ct*HPF-125 or 36% by *Ct*HPF-119, respectively (Figure 7b[1,2]). Furthermore, when both *Ct*HPF-125 and *Ct*HPF-119 were added to the in vitro transcription-translation reaction mixture containing logarithmic growth phase *Ct*-ribosomes, translation activity was inhibited by approximately 58% (Figure 7b[2]).

2.9 | Mutant *Ct*HPF-125 lacking the central six amino acids (Δ50–55) has a stronger translation inhibitory effect than the wild-type protein or *Ct*HPF-125 (Δ106–125)

*Ct*HPF-125 has two characteristic features that differentiate it from HPFs of other bacteria; the first is the presence of an additional six amino acid residues (50–55) in the central region between the β2 and β3 sheets, and the

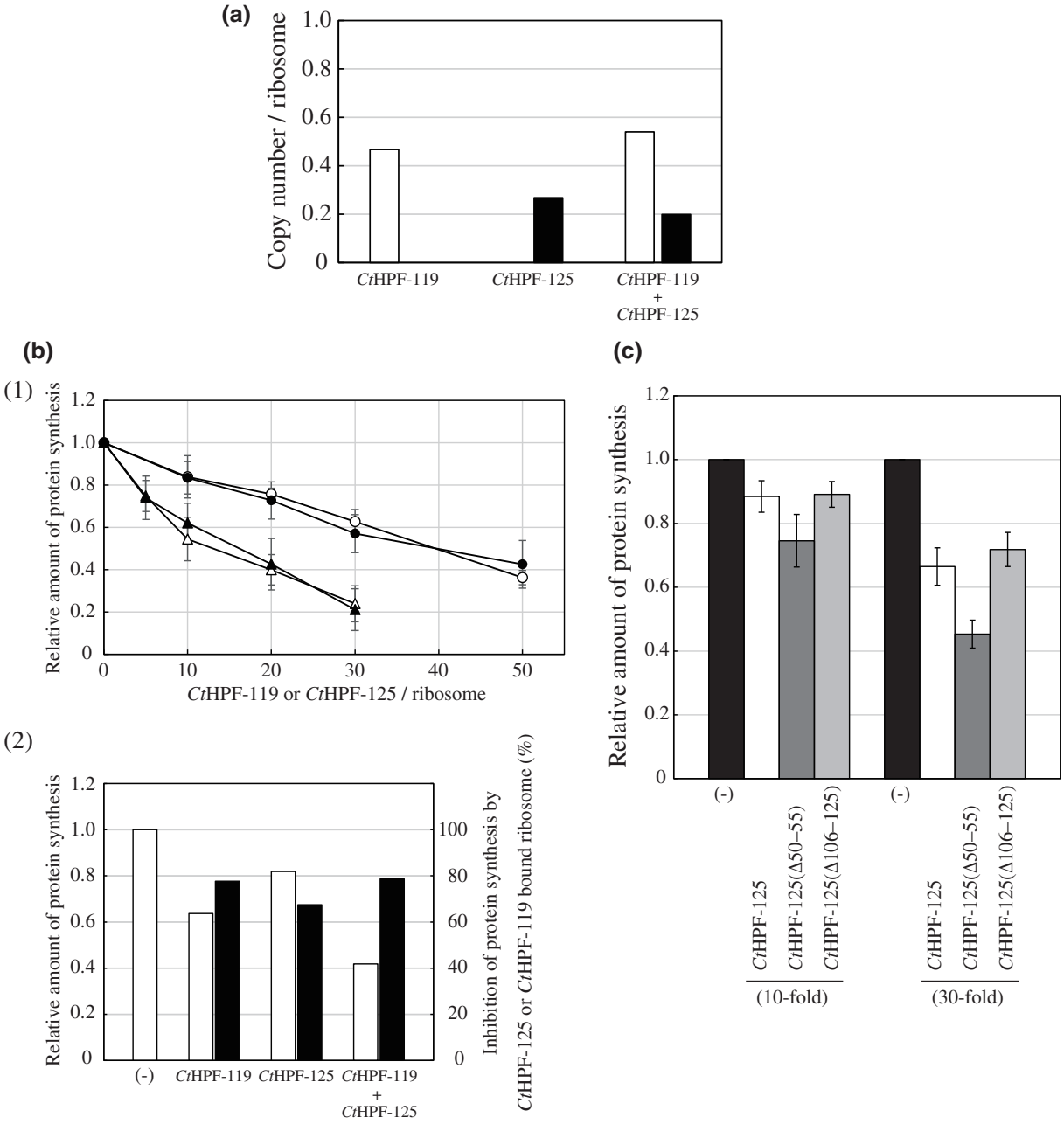
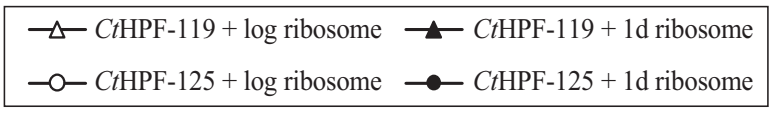


FIGURE 7 *Ct*HPF-119 and *Ct*HPF-125 bind to *Ct*-ribosomes and inhibit translation in vitro. (a) In vitro ribosome binding assay of CtHPF-119 and CtHPF-125. HSRs were prepared from C. testosteroni cells cultured for 1 day. The HSRs were then incubated with purified CtHPF-119 (10-fold) and/or CtHPF-125 (10-fold) at 30°C for 30 min. The mixture was centrifuged to collect the ribosomes, and the r-proteins were separated by RFHR 2D PAGE. The copy numbers of CtHPF-119 and CtHPF-125 per ribosome were then measured. (b) In vitro transcription-translation activity assays using *Ct*HPF-125 and *Ct*HPF-119. (1) Assay mixtures contained *Ct*-ribosomes prepared from cells in the logarithmic phase (open circles or triangle) or the stationary phase (filled circles or triangle) and varying amounts of *Ct*HPF-125 or *Ct*HPF-119 (10-, 20-, 30-, or 50-fold per ribosome). Data are represented as the mean ± SD of at least three independent experiments.



(2) Assay mixtures contained *Ct*-ribosomes prepared from cells in the logarithmic phase and 10-fold (per ribosome) amounts of *Ct*HPF-119 and/or *Ct*HPF-125. The left y-axis (white box). shows translation activity relative to that without the protein's addition. The right y-axis (black box) shows the degree (percent) of inhibition assuming to be inhibited by *Ct*HPF-125 and/or *Ct*HPF-119 bound to ribosome.
(c) In vitro transcription-translation assays using *Ct*-ribosomes and wild-type *Ct*HPF-125 or the *Ct*HPF-125(Δ50–55) or *Ct*HPF-125(Δ106–125) mutant (10-fold or 30-fold per ribosome). Data are represented as the mean ± SD of at least three independent experiments.

second is a disordered C-terminal sequence (106–125), which is not present in *Ec*HPF (Figure 2, Figure S1a). To examine the roles of these two domains in the inhibitory effect of *Ct*HPF-125 on translation activity, we generated and overexpressed YB1035 [KRX (pTXB1-*Ct-hpf*-125 (Δ50–55))] and YB1036 [KRX (pTXB1-*Ct-hpf*-125(Δ106–125))] deletion mutants in *E. coli*. and purified both proteins as described in the Section 4. When added to in vitro transcription-translation assay reaction mixtures at 10-fold or 30-fold per ribosome, the inhibitory activity of *Ct*HPF-125(Δ50–55) was 1.8- or 2.8-fold stronger than that of wild-type *Ct*HPF-125, respectively. By contrast, the inhibitory activity of *Ct*HPF-125(Δ106–125) was similar to that of the wild-type protein (Figure 7c). These results indicate that the six amino acids between the β2 and β3 sheets of *Ct*HPF-125 contribute to relief of the inhibition of translation activity in vitro, whereas the C-terminal region of *Ct*HPF-125 is unlikely to play a role.

2.10 | *Ct*HPF-125 cannot form 100S ribosomes and the 70S ribosome of *C. testosteroni* C*thpf* mutant cells decreases suddenly than that of wild type cells in the stationary phase

In response to stress conditions such as nutrient depletion (i.e., stationary phase culture), the inactive 100S (70S dimer) ribosomes in *E. coli* (representing Gammaproteobacteria) are formed by *Ec*RMF and *Ec*HPF (short HPF) (Wada et al., 1990; Yoshida & Wada, 2014). Many other bacteria show a similar stress response but utilize long HPF (Ueta et al., 2010, 2013). The ribosome binding sites of *Ct*HPF, *Ec*HPF, and long HPF are similar; however, the C-terminal regions of these proteins differ in both length and amino acid content (Figure 2, Figure S1a). To investigate whether *Ct*HPF can promote the formation of 100S ribosomes, CR fractions were prepared from *C. testosteroni* cells in the logarithmic growth and stationary growth phases. The cells were disrupted using quartz sand, and the CE was ultracentrifuged without a sucrose cushion. Subsequently, the CR fraction was collected from the pellet and subjected to 5%–20% sucrose density gradient (SDG) centrifugation. The analysis revealed that 70S ribosomes, but not 100S ribosomes, were detected in the CR fraction of cells in the logarithmic growth phase (5 h) as well as in that of cells in the stationary phase (10 h, 15 h, 1 day, 2 days, 3 days) (Figure 8a), in which *Ct*HPF-125 is expressed (Figure 4a). These findings indicate that *Ct*HPF-125 is unable to form 100S ribosomes in the stationary phases.

A similar SDG analysis of CR fractions prepared from *C. testosteroni* cells exposed to heat shock (37°C) or cold shock (20°C) also detected 70S ribosomes but no 100S ribosomes (Figure S3).

The yield of the CR fraction of *E. coli* cells, which contains mainly ribosomes, decreased by approximately 62% following transition from the logarithmic growth phase (5 h) to the stationary phase (24 h), and then remained constant throughout the 3 days analysis period (Figure 8b). By contrast, the yield of the CR fraction of *C. testosteroni* wild type and mutant cell continued to decrease after entering the stationary phase and dropped to approximately 18% and 11% of that of the logarithmic growth phase after 3 days and the reduction was more pronounced in the mutant strain than in the wild-type strain (Figure 8b). Next, we investigated whether 70S particles in the CR fraction of *C. testosteroni* wild type and mutant cells are retained stably in stationary phase. The cells were cultured and sampled at various times (5 h, 10 h, 1 day, 2 days, and 3 days), and the CEs were ultracentrifuged without a sucrose cushion. Subsequently, the CR fractions were collected as the pellet and analyzed by 5%–20% SDG centrifugation. In the logarithmic growth phase, 70S ribosomes accounted for 67% and 72% of the CR fraction in wild type and the mutant cells, respectively but this percentage decreased to 50% and 28% after 3 days, and the percentage of the upper fraction containing degradation products of ribosome increased after 1 day (Figure 8a,c[1,2]). Next, to remove low MW RNAs, the CE was ultracentrifuged with a 30% sucrose cushion. The CR was collected and analyzed by 5%–20% SDG centrifugation. In this experiment, the 30S and 50S subunits were not observed by SDG centrifugation with 15 or 6 mM Mg^{2+} in the CR fractions of cells cultured for 1 day (Figure 8d). These findings suggest that *Ct*-ribosomes do not dissociate into the 30S and 50S subunits in the stationary phase, despite a reduction in the number of ribosomes per cell after culture for 1 day. Furthermore, these results suggests that *Ct*-ribosomes do not decompose during culture for up to 1 day。 However, in the mutant strain, 70S particle degradation is higher than in the wild-type strain from 1 day, and the decay progresses significantly over the next 3 days. This suggests that *Ct*HPF-125 is responsible for preventing 70S from degradation in the stationary phase.

2.11 | Long HPF promotes the formation of 100S ribosomes from 70S ribosomes in *C. testosteroni* ATCC11996

Betaproteobacteria such as *C. testosteroni* do not have the hibernation factors found in other bacteria (long HPF or RMF plus short HPF) which form 100S ribosome, but have two distinct *Ct*HPFs and express one (*Ct*HPF-125)

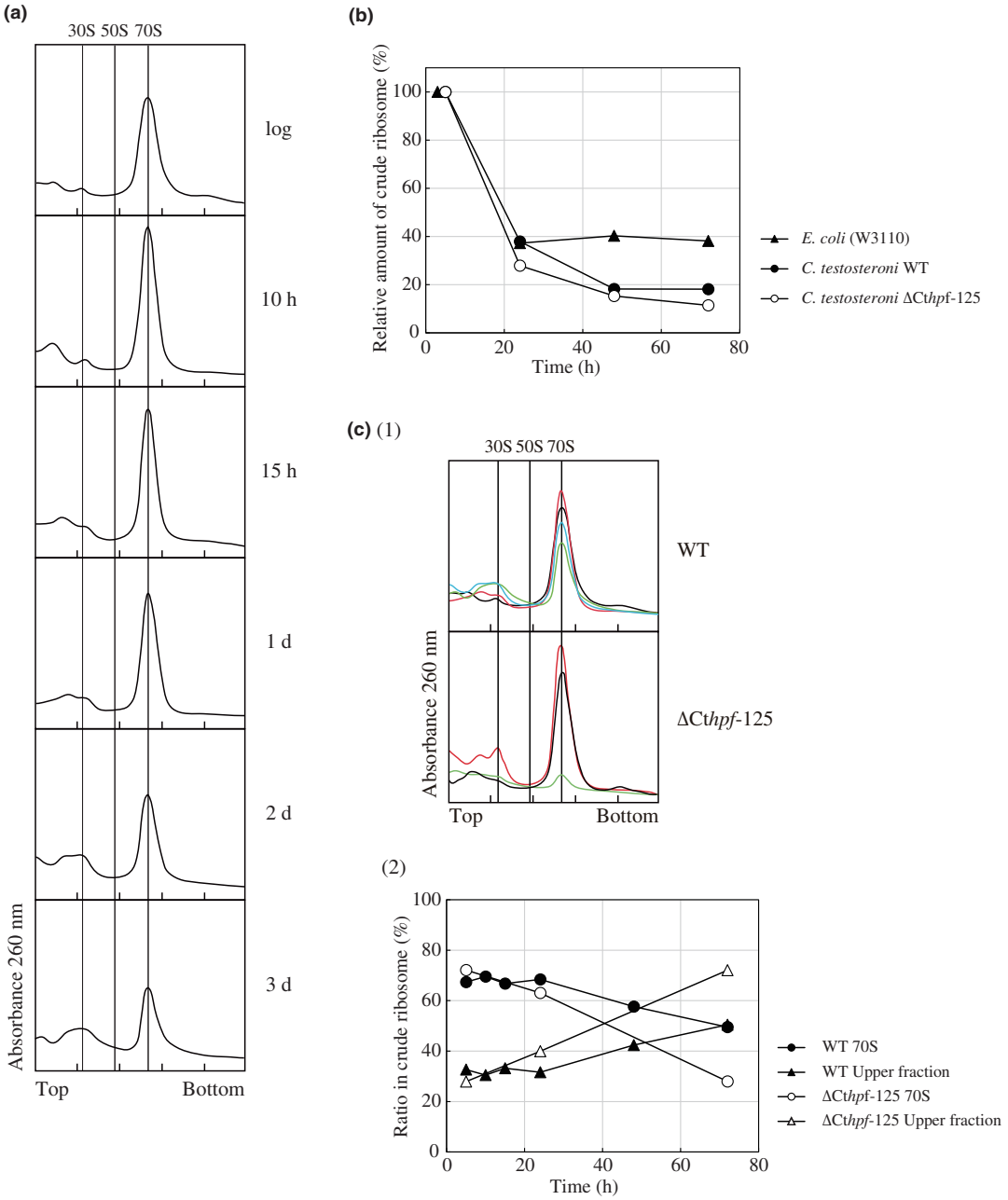


FIGURE 8 *C. testosteroni* does not form 100S ribosomes in the logarithmic growth or stationary phase. (a) The ribosome profiles of CR fractions (60 pmol) prepared from *C. testosteroni* cells in the logarithmic phase (5 h) and stationary phase (10 h, 15 h, 1 day, 2 days, and 3 days). The CR fractions were analyzed by 5%–20% SDG centrifugation. The direction of the 5%–20% SDG is from left (top) to right (bottom). (b) The ribosome yields of *C. testosteroni* ATCC11996 wild, C*thpf*-125 mutant cells and *E. coli* W3110 cells in the logarithmic and stationary phases. The amount of ribosomes in each strain was estimated based on the absorbance of the CR fraction normalized to the cell weight. The y-axis shows the amount of ribosome relative to that of the logarithmic phase. Closed circle, open circle, and closed triangle show *C. testosteroni* wild, C*thpf*-125 mutant cells and *E. coli* W3110 cells, respectively. (c) (1) The stability of 70S ribosomes in the CR fraction of *C. testosteroni* wild and C*thpf*-125 mutant cells. CR fractions were prepared from *C. testosteroni* (WT) and the C*thpf*-125 mutant cells in the logarithmic phase (5 h; black) and stationary phase (1 day; red, 2 days; blue, and 3 days; green). The CR fractions (60 pmol) were analyzed by 5%–20% SDG centrifugation. The profiles were shown. The direction of the 5%–20% SDG is from left (top) to right (bottom). Vertical axis shows absorbance at OD 260. (2) The percentages of the CR fraction represented by 70S ribosomes and the upper fraction (fraction excluding 70S) were calculated from the ribosome profiles shown in (c) (1). Closed circle, open circle, closed triangle and open triangle show wild 70S, C*thpf*-125 mutant 70S, wild upper fraction and C*thpf*-125 mutant upper fraction, respectively. Vertical axis shows 70S ratio in crude ribosome as percentages (%). (d) The ribosome profiles of CR fractions prepared from *C. testosteroni* cells cultured for 1 day. The CR fractions were prepared by ultracentrifugation using a 30% sucrose cushion and were analyzed by 5%–20% SDG centrifugation with different Mg^{2+} concentrations (6 or 15 mM). The direction of the 5%–20% SDG is from left (top) to right (bottom).

of two (Table S2). As described above, 70S ribosomes, but not 100S ribosomes, were detected in *C. testosteroni* cells during the logarithmic growth and stationary phases (Figure 8a). Therefore, we investigated whether ribosomes from *C. testosteroni* can form 100S ribosomes in vitro in the presence of long HPF (*Lp*HPF) or *Ec*RMF plus *Ec*HPF. Although *Ec*RMF and *Ec*HPF were able to bind to *E. coli* 70S ribosomes which were prepared from *rmf*, *hpf* and *yfiA* deleted cells and form 100S ribosomes, they were unable to form 100S ribosomes from *C. testosteroni* 70S ribosomes (*Ct*-70S ribosomes) (Figure 9a). Notably, *Ec*HPF was able to bind to *Ct*-70S ribosomes, whereas *Ec*RMF was not (Figure 9a). On the other hand, the long HPF homolog of *L. paracasei* (*Lp*HPF) was able to bind to *Ct*-70S ribosomes and formed *Ct*-100S ribosomes (Figure 9b). These findings indicate that, unlike *Ct*HPF-125, ancestral-type long HPF is able to promote the dimerization of *Ct*-70S ribosomes to form *Ct*-100S ribosomes.

3 | DISCUSSION

This study investigated the functions of two HPF homologs in the Betaproteobacteria class, using *C. testosteroni* ATCC11996 as a representative species. The genome of *C. testosteroni*, which belongs to the *Comamonas* genus (*Comamonadaceae* family, *Burkholderiales* order, Betaproteobacteria class), is present. two HPF homologs: *Ct*HPF-125 and *Ct*HPF-119, which contain 125 and 119 amino acid residues, respectively. While *Ct*HPF-119 orthologs are universally present in all Betaproteobacteria genomes

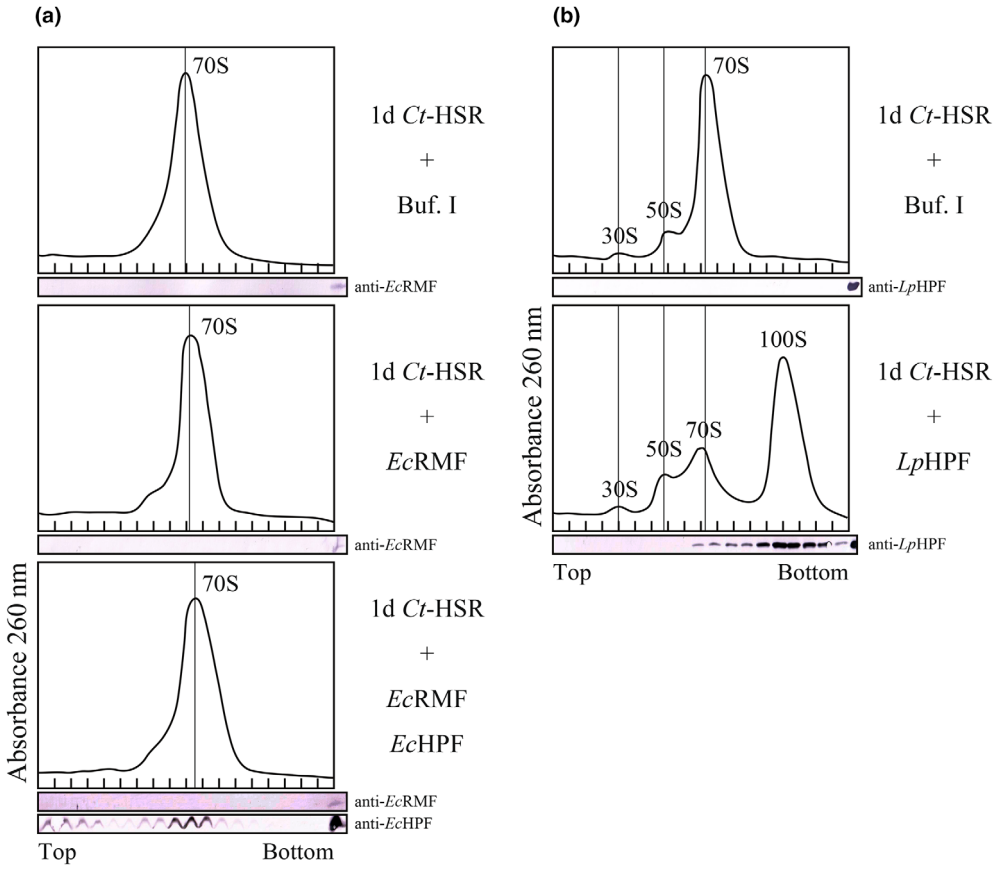


FIGURE 9 Ancestral-type long HPF promotes the dimerization of *Ct*-70S ribosomes to form *Ct*-100S ribosomes. (a) The ribosome profiles of 5%–20% SDG centrifugation after incubation *Ct*-HSRs and *Ec*RMF and/or *Ec*HPF. Ct-HSRs were prepared from *C. testosteroni* cells cultured for 1 day and then incubated with Buffer I, *Ec*RMF, and/or *Ec*HPF at 37°C for 30 min. The reaction mixtures were analyzed by 5%–20% SDG centrifugation. The direction of the 5%–20% SDG is from left (top) to right (bottom). The images below the graphs show western blot analyses of the corresponding fractions using an anti-*Ec*RMF or anti-*Ec*HPF antibody. Purified *Ec*RMF (1.9 μg) and *Ec*HPF (3.2 μg) protein were loaded as controls. (b) The ribosome profiles of 5%–20% SDG centrifugation after incubation *Ct*-HSRs and *Lp*HPF. Ct-HSRs were prepared as described in (a) and dissociated as described in the Section 4. The reaction mixture containing *Lp*HPF and dissociated *Ct*-HSR was incubated at 37°C for 30 min, and then the ribosome fractions were collected and analyzed by 5%–20% SDG centrifugation. The direction of the 5%–20% SDG is from left (top) to right (bottom). The images below the graphs show western blot analyses of the level of *Lp*HPF protein in the corresponding fractions. Purified *Lp*HPF (2.6 μg) protein was loaded as a control.

sequenced so far, Betaproteobacteria harboring *Ct*HPF-125 are confined to the *Comamonadaceae* family, some members of the *Burkholderiaceae* family, some members of the *Oxalobacteraceae* family, *Paenalcaligenes hominis*, *Nitrosomonas communis*, *Thiobacillus denitrificans*, and *Azospira oryzae* (Table S2). An alignment analysis revealed that the amino acid homology of *Ct*HPF-119 to *Ec*HPF is higher than that of *Ct*HPF-125 to *Ec*HPF (Figure 2, Figure S1).

We found that *Ct*HPF-125 was expressed in stationary phase cells but not in logarithmic growth phase cells (Figures 3 and 4a,b). *Ct*HPF-125 existed stably in the PRS fraction (Figure 4a[3],b), whereas HPFs from *E. coli* (*Ec*HPF), *S. aureus* (*Sa*HPF), and *L. paracasei* (*Lp*HPF) are bound to ribosomes, exist in the CR fraction but are not found in the PRS fraction (Ueta et al., 2010, 2013; Yoshida et al., 2019). Therefore, it indicates that *Ct*HPF-125 exists stably in a free state in addition to being bound to the ribosome in the cell. When not bound to ribosomes, *Ec*HPF, *Sa*HPF, and *Lp*HPF are unstable and may be degraded by proteases. We hypothesize that *C. testosteroni* does not contain such proteases or that *Ct*HPF-125 is resistant to protease degradation. We don't know if free HPF has any other functions. RFHR 2D PAGE and western blotting analyses did not detect *Ct*HPF-119 in the CR, PRS, or CE fraction of cells in the logarithmic growth or stationary phase (Figures 4a and 5b). Nonetheless, it would be worth examining the expression of *Ct*HPF-119 in other culture conditions.

The ribosome binding ability of *Ct*HPF-125 was lower than those of other HPF homologs, including *Ct*HPF-119, which have nearly identical binding amino acid sequences to ribosomes. Alignment of the amino acid sequences of *Ct*HPF-125, *Ct*HPF-119, *Ec*HPF, and *Lp*HPF showed that two (the first and sixth) of six amino acids involved in ribosome binding differ in *Ct*HPF-125 (E24, T97) compared with *Ct*HPF-119 (K23, R92), *Ec*HPF (K23, K87), and *Lp*HPF (R24, K91) (Figure 2; red shading), representing a change from positively charged amino acids (K or R) to negatively charged (E24) or hydrophilic (T97) amino acids in *Ct*HPF-125. The lower ribosome binding ability of *Ct*HPF-125 may be a result of the replacement of these two basic amino acids.

YfiA (*Ec*YfiA) which is a homolog of *Ec*HPF is induced during the cold shock stress and the stationary phase (Agafonov et al., 2001; Maki et al., 2000). However, *Ct*HPF-125 and *Ct*HPF-119 were not expressed in *C. testosteroni* cells exposed to cold or heat stress (Figure 6). *Ct*HPF-119 were not expressed in the *Ct*HPF-125 deletion mutant. As *Ct*HPF-119 are universally present in all Betaproteobacteria genomes sequenced so far, additional experiments are required to investigate whether these proteins are expressed in other stress conditions. We will examine the function of *Ct*HPF-119 in Betaproteobacteria that have only *Ct*HPF-119.

We also investigated the functions of *Ct*HPF-125 and *Ct*HPF-119 in translation in vitro. The addition of *Ct*HPF-125 or *Ct*HPF-119 to an in vitro transcription-translation system dose-dependently inhibited the translational activity (Figure 7b[1,2]). Notably, the inhibitory activity of *Ct*HPF-119 (36%) was higher than that of *Ct*HPF-125 (18%). In addition, an in vitro binding analysis revealed that 0.47 copies of *Ct*HPF-119 and 0.27 copies of *Ct*HPF-125 bind to each ribosome (Figure 7a), which corresponds to the difference between the translation inhibitory activities of these two proteins. Furthermore, when both *Ct*HPF-125 and *Ct*HPF-119 were added to the in vitro transcription-translation reaction mixture containing logarithmic growth phase *Ct*-ribosomes, translation activity was inhibited by approximately 58% (Figure 7b[2]). Assuming that *Ct*HPF-125 (0.27/ribosome), *Ct*HPF-119 (0.47/ribosome), or *Ct*HPF-125 plus *Ct*HPF-119 (0.74/ribosome) bound to Ct ribosome inhibit in vitro translation activity, these inhibition is about 70%–80% (Figure 7b[2] black box). Overall, these results suggest that *Ct*HPF-125 and *Ct*HPF-119 inhibit translation in vitro by binding to ribosomes.

Two deletion mutants were constructed to characterize the region of *Ct*HPF-125 involved in the inhibition of translation activity. The *Ct*HPF-125(Δ50–55) mutant harboring a deletion of the six amino acids between the β2 and β3 sheets had a stronger translational inhibitory effect than the wild-type protein. On the other hand, the inhibitory effect of a deletion mutant lacking the 20 C-terminal amino acids (*Ct*HPF-125(Δ106–125)) was similar to that of the wild-type protein (Figure 7c). The copy numbers of *Ct*HPF-125, *Ct*HPF-125(Δ50–55), and *Ct*HPF-125(Δ106–125) bound to each ribosome were comparable (Figure S4). These results suggest that the central region of *Ct*HPF-125 may attempt to diminish the effect of the translation inhibition, whereas the C-terminal region does not. A previous structural and biochemical analysis of *Ec*YfiA revealed that it fills the tRNA- (A site and P site) and mRNA-binding channel of the 30S subunit and impairs initiation factor-dependent binding of fMet-tRNA to ribosomes at 16°C (Vila-Sanjurjo et al., 2004). Since *Ec*YfiA and *Ct*HPF-125 share similar ribosome binding sequences (Figure 2), the location of *Ct*HPF-125 on the ribosome may be the same as that of *Ec*YfiA (Figure 2). Therefore, the central region of *Ct*HPF-125 may locate close to the A-site (tRNA-binding site) of the 30S subunit and regulate translation inhibition. Further experiments are required to elucidate the mechanism of translational inhibition by *Ct*HPF-125.

Bacteria having long HPF or RMF and short HPF as hibernation factor, form 100S ribosome but

Betaproteobacteria such as *C. testosteroni* cannot form 100S ribosome by *Ct*HPF-125 under stress conditions. To determine whether this difference results in *Ct*-70S ribosome, we examined the ability of *Ct*-70S ribosomes to form 100S ribosomes in vitro in the presence of *Lp*HPF (long HPF) or *Ec*RMF and *Ec*HPF. Although *Ec*HPF (but not *Ec*RMF) bound to the *Ct*-70S ribosome (Figure 9a), the protein was unable to promote the formation of *Ct*-100S ribosomes. By contrast, *Lp*HPF bound to *Ct*-70S ribosomes and induced the formation of 100S ribosomes (Figure 9b). Therefore, although *Ct*HPF-125 cannot promote the formation of *Ct*-100S ribosomes, *Ct*-70S ribosomes have the potential to dimerize and form *Ct*-100S ribosomes in the presence of ancestral-type long HPF, as was observed in *E. coli* ribosome which delete *Ec*RMF, *Ec*HPF, and *Ec*YfiA (Ueta et al., 2013). Lack of the C-terminal domain of long HPF, which is essential to the HPF-HPF hydrophobic interaction required for 100S formation (Usachev et al., 2020), may explain why *Ct*HPF-125 is able to bind to ribosomes but cannot induce 100S ribosome formation.

In *E. coli*, *Ec*RMF (55 amino acids) is required for prolonged survival during nutrient starvation conditions, whereas *Ec*HPF (95 amino acids) and *Ec*YfiA are not (Ueta et al., 2005; Yamagishi et al., 1993). *Pseudomonas aeruginosa* has two genes coding *Pa*RMF (70 amino acids) and *Pa*HPF (102 acids), corresponding to *Ec*RMF and *Ec*HPF, and has not *yfiA* gene. *Pa*HPF is necessary for prolonged survival under nutrient-limited conditions, whereas *Pa*RMF is not (Akiyama et al., 2017). By our experiments, *Pseudomonas fluorescens* also have two genes of *Pf*HPF (102 amino acids; 77% identity with *Pa*HPF) and *Pf*RMF (70 amino acids; 80% identity with *Pa*RMF), and the genes encoding the HPF and RMF proteins of *P. aeruginosa* or *P. fluorescens* are located at the same genomic position in each species. HPF and RMF of two strains are similar. In *P. fluorescens* cells, an RFHR 2D PAGE analysis detected *Pf*HPF but could not *Pf*RMF in stationary phase. In addition, 100S ribosomes were not detected in the stationary phase cells (our unpublished data). It suggests that *Pf*HPF cannot form 100S ribosome but have the function of prolonged survival. Similarly, *Pa*RMF may not be expressed in *P. aeruginosa*. The strains belong to Gamma Proteobacteria, Pseudomonadaceae but could not form 100S ribosome by *Pf*RMF and *Pf*HPF (Figure S5).

We isolated for the first time a deletion mutant strain of *Ct*HPF-125 gene from *C. testosteroni*. In the deletion mutant, the yield of ribosomes was lower than wild type strain and 70S ribosomes were collapsed earlier than in wild type strain (Figure 8b,c). Growth (colony former) of the mutant strain in the stationary phase also decreased faster than wild type strain (Figure 1c). This suggests that *Ct*HPF-125 is a hibernation factor of *C. testosteroni*. *Ct*HPF-125 does not produce 100S (Figure 8a), as *Pa*HPF of *P. aeruginosa* or *P. fluorescens*, but instead binds to 70S, thereby interrupting translation during stress and allowing the strain to survive.

Here, we found that the numbers of ribosomes per cell in *C. testosteroni* and *E. coli* decreased suddenly during transition from the logarithmic growth phase to the stationary phase. Subsequently, the ribosome number of *C. testosteroni* continued to decrease, whereas that of *E. coli* remained fairly constant throughout the 3 days observation period (Figure 8b). Furthermore, in *C. testosteroni*, 70S ribosomes accounted for a large percentage (about 70%) of the CR fraction, and their level was constant in cells cultured for up to 1 day but then decreased gradually with extended culture (Figure 8c). The 70S ribosomes did not dissociate into the 30S and 50S subunits in exponential growth or stationary phase cells, and the level of fractions smaller than 30S increased gradually after culture for 1–3 days (Figure 8a,c). The colony former of *C. testosteroni* increased from 3.5×10^8 cells/mL after culture for 8 h to 1.4×10^9 cells/mL after culture for 2 days, and then decreased sharply after 3 days (Figure 1c). In the early stationary phase, despite the decrease in the number of ribosomes per cell, the cells continued to proliferate for two generations. This suggest that ribosomes are not newly synthesized and not yet collapsed in the early stationary phase, the existing ribosomes are distributed, and the number of ribosomes per cell decreases. The life of *C. testosteroni* is shorter than that of *E. coli*, which may be attributed to the fact that *Ct*HPF-125 cannot form 100S ribosomes in contrast to *E. coli* and binds weakly to 70S ribosomes.

Recent studies have shown that *E. coli* hibernation factors (HPF and RMF) and *Sa*HPF from *S. aureus* prevent ribonuclease-mediated degradation of rRNA to protect complete ribosomes, thereby contributing to ribosome particle homeostasis and efficient resumption of logarithmic growth after nutrient replenishment (Lipońska & Yap, 2021; Prossliner et al., 2021). Ribosomes of *B. subtilis* cells lacking the *Bshpf* gene lose r-proteins S2 and S3 in the stationary phase (Feaga et al., 2020), and ribosomes of *P. aeruginosa* cells lacking the *Pahpf* gene lose r-proteins L5 and S13 (Theng et al., 2020). These findings suggest that *Bs*HPF and *Pa*HPF bind to ribosomes and protect against ribosome collapse during nutrient deficient conditions, ensuring that ribosomes are ready for use when the stress is removed, and that translation can restart rapidly without new ribosome synthesis.

To summarize, this study demonstrates that *Ct*HPF-125, one of two HPF homologs in *C. testosteroni*, is expressed specifically in the stationary phase. Together

with the results of several other bacterial HPF studies, the data presented here suggest that binding of *Ct*HPF-125 to the ribosome stops translation and protects against 70S ribosome degradation, enabling *C. testosteroni* cells to restart translation without the synthesis of new ribosomes when stress (such as nutrient starvation) is removed. *Ct*HPF-125 may be involved in cellular translational regulation as hibernation factor as HPF of other bacteria; however, as it binds to ribosomes weakly, ribosome collapse likely continues during the late stationary phase, and *C. testosteroni* cells die at a faster rate than *E. coli*.

Based on the phylogenetic tree, we summarized the transition of the hibernation factor and the 100S ribosome formation (Figure S5).

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and plasmids

The *C. testosteroni* ATCC11996 (NBRC 14951^T) strain was used. The *Cthpf*-125 and *Cthpf*-119 genes were amplified by PCR from genomic DNA isolated from *C. testosteroni* ATCC11996. The following primers were used: 5′-NdeI-*Cthpf*-125-F and 3′-SapI-*Cthpf*-125-R, or 5′-NdeI-*Cthpf*-119-F and 3′-SapI-*Cthpf*-119-R (Table S1). The amplified products were cloned into the pTXB1 expression vector (IMPACT System; New England Biolabs, Inc., Ipswich, MA), and the resulting plasmids (pTXB1-*Cthpf*-125 and pTXB1-*Cthpf*-119) were transformed into the *E. coli* KRX strain (Promega, Madison, WI) to generate the YB1035 [KRX (pTXB1-*Cthpf*-125)] and YB1036 [KRX (pTXB1-*Cthpf*-119)] strains, respectively. The cloned *Cthpf*-119 and *Cthpf*-125 products were confirmed by DNA sequencing. The sequence of cloned *Cthpf*-119 was identical to that of *Cthpf*-119 from *C. testosteroni* ATCC11996 (WP_003072415.1). Four independent clones of *Cthpf*-125 had a single nucleotide replacement (A369C) compared with *Cthpf*-125 from *C. testosteroni* ATCC11996 (WP_003060358.1).

To generate the C-terminal deletion mutant of *Cthpf*-125, the ORF of *Cthpf*-125(Δ106–125) was amplified using genomic DNA isolated from *C. testosteroni* as a template and the following PCR primers: 5′-NdeI-*Cthpf*-125-F and 3′-SapI-*Cthpf*-125(Δ106–125)-R (Table S1). The central deletion mutant of *Cthpf*-125 was generated by PCR using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio Inc., Shiga, Japan), pTXB1-*Cthpf*-125 as a template, and the following PCR primers: *Cthpf*-125(Δ50–55)-1 and *Cthpf*-125(Δ50–55)-2 (Table S1). The DNA sequences of the cloned deletion mutants were confirmed by sequencing. The pTXB1-*Cthpf*-125(Δ106–125) and pTXB1-*Cthpf*-125(Δ50–55) plasmids were transformed into the *E. coli* KRX strain to generate the YB1037 [KRX (pTXB1-*Cthpf*-125(Δ106–125))] and YB1038 [KRX (pTXB1-*Cthpf*-125(Δ50–55))] strains, respectively.

4.2 | Medium and growth conditions

The *C. testosteroni* ATCC11996 strain was grown by shaking at 30°C in 802-medium containing 1% Hipolypepton (FUJIFILM-Wako, Osaka, Japan), 0.2% yeast extract (Difco, BD Biosciences Advanced Bioprocessing 50 NW 176 Street, Miami, USA) and 0.1% $MgSO_4/7H_2O$ (pH 7.0). Cell growth was estimated by measuring the cell turbidity using a Klett–Summerson photoelectric colorimeter (Bel-Art Products, Wayne, NJ, USA) with a green filter (#54). Cells were harvested in the logarithmic and stationary phase, and stored at −80°C until use. Cell growth on 802-medium agar plates was determined by monitoring the colony size or by counting colony numbers after incubation at 30°C for 2 days.

4.3 | Construction of the C*thpf*-125 deletion mutant (ΔC*thpf*-125)

C*thpf*-125 deletion mutants of *C. testosteroni* (NBRC 14951^T, ATCC11996) were constructed by modifications of a rapid streamlined method (Huang & Wilks, 2017). pEX18Tc plasmid was replaced by the shuttle vector pMADcm (Tsai et al., 2011), which is designed for efficient allelic replacement in Gram positive bacteria (Arnaud et al., 2004) and cannot replicate stably at 30°C in *C. testosteroni*. The properties were useful to select the genes integrated by homologous recombination into chromosomes. The chloramphenicol gene of pMADcm was cut out with SalI and BglII and replaced with the fragment (SalI-C*thpf*-125-Up-tet-C*thpf*-125-Down BglII), which was replaced the C*thpf*-125 gene of the host with tetracycline gene of pMADtet (Tsai et al., 2011). Three fragments were amplified by PCR with the following primers (Table S2) and were sequentially ligated using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs).

[tet gene]
C*thpf*-125-Up-tet-F: GAGGATTTGCTAAGTGATGAAATACTGA
C*thpf*-125-Down-tet-R: AACGCTTCATCTGTTATAAAAAAGGAT
[upstream region]
SalI-C*thpf*-125-Up-F: GATCGTCGACCCGGCGAAATCTGGATTC

tet-C*thpf*-125-Up-R: TCATCACTTAGCAAATCCTCC TAGTCAC
[downstream region]
tet-C*thpf*-125-down-F: TTTATAACAGATGAAGCGT TTTTGGCTTC
BglII-C*thpf*-125-Down-R: CGAGATCTGCAACGATA CCGCCATCAC

The constructed fragment was amplified by PCR and digested by SalI and BglII, and ligated into SalI-BglII sites of pMADcm to generate the pMAD Up gene region (500 bp)-tet(ΔC*thpf*-125)-Down gene region(505 bp), and transformed to *E. coli* NEB-5 alpha strain (New England Biolabs) and the tetracycline-resistant transformant was isolated and the plasmid DNA was prepared. The inserted fragment of the target plasmid was confirmed by PCR and sequencing. The target plasmid DNA was introduced into *C. testosteroni* cells by electroporation. The tetracycline-resistant ΔC*thpf*-125 deletion mutant was isolated using 802-medium plate with 10 μg/mL tetracycline. The absence of the C*thpf*-125 gene in the deletion mutant cells was confirmed by colony PCR using the following primer pairs and sequencing the PCR fragment.

del-F: GATGATTGAGGTCAGCCCTG
del-R: GTCTGCTTCGAAGTGATAGG

4.4 | Preparation of the CR, PRS, and CD fractions

CRs were prepared from CEs according to the method of Noll et al. (1973) with slight modifications (Horie et al., 1981). Harvested cells were ground with similar volumes of quartz sand (FUJIFILM-Wako, Osaka, Japan), and the disrupted cells were extracted with buffer I [20 mM Tris–HCl (pH 7.6), 15 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol]. The homogenate was centrifuged at 9000*g* for 15 min at 4°C. The supernatant was saved, and the pellet was resuspended in buffer I. Subsequently, the suspension was centrifuged again under the same conditions, and the resulting pellet was collected as the CD fraction. The combined supernatants (CE) were layered onto a 30% sucrose cushion in buffer I and centrifuged in a 55.2 Ti rotor (Beckman, Fullerton, CA, USA) at 206,000*g* for 3 h at 4°C. To minimize dissociation of *Ct*HPF-125 from *C. testosteroni* ribosomes, the CE was centrifuged without a sucrose cushion. The pellet was resuspended in buffer I and used as the CR fraction, and the supernatant was used as the PRS.

4.5 | Preparation of HSRs

HSRs were prepared as described by Horie et al. (1981). Briefly, the CR fraction was resuspended in buffer II [20 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate, 1 M ammonium acetate, and 6 mM 2-mercaptoethanol] and mixed for 1 h at 4°C. Subsequently, the high-salt-washed suspension (20 mL) was layered onto a 30% sucrose cushion in buffer II (10 mL) and centrifuged in a 55.2 Ti rotor (Beckman) at 206,000*g* for 4 h at 4°C. The pellet was resuspended in buffer I and dialyzed against the same buffer overnight. This suspension was used as the HSR fraction.

4.6 | Preparation of 30S and 50S subunits

HSRs were suspended in dissociation buffer I [20 mM Tris–HCl (pH 7.6), 1 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol] and dialyzed against the same buffer overnight. The sample was then layered onto a 10%–40% SDG in dissociation buffer I and centrifuged in a 45 Ti rotor (Beckman) at 20,000*g* for 19 h at 4°C. The gradient was fractionated, and the absorbance of each fraction was measured using a UV-1800 spectrometer (Shimadzu, Kyoto, Japan) at 260 nm. The 30S and 50S fractions were collected, and each subunit was pelleted by centrifugation in a 55.2 Ti rotor (Beckman) at 206,000*g* for 4 h at 4°C. Each pellet was resuspended in buffer I.

4.7 | Analysis of ribosomes by SDG centrifugation

Each ribosome sample was layered onto a 5%–20% SDG (12 mL) in buffer I and centrifuged in a SW 40 Ti rotor (Beckman) at 25,000*g* for 20 h at 4°C. The SDGs were prepared using a Gradient Mate 6T instrument (BioComp Instruments, Fredericton, NB, Canada). The absorbance of each fraction was measured at 260 nm using a flow cell in a UV-1800 spectrometer (Shimadzu).

4.8 | RFHR 2D PAGE

C. testosteroni ATCC11996 proteins were prepared by the acetic acid method (Hardy et al., 1969). A one-tenth volume of 1 M $MgCl_2$ and two volumes of acetic acid were added to the protein solution, and the mixture was stirred for 1 h at 0°C. After centrifugation at 10,000 g for 10 min, the supernatant was dialyzed three times against 2% acetic acid (the volume of the dialysis buffer was 300-fold

larger than the volume of the sample) for 24 h. The proteins were lyophilized and stored at −80°C until use. The protein solution (2 mg of protein in 100 μL of 8 M urea containing 0.2 M 2-mercaptoethanol) was analyzed by RFHR 2D PAGE as described previously (Wada, 1986a, 1986b), with some slight modifications (Ueta et al., 2010). RFHR 2D PAGE was improved on the 2D PAGE method of Kaltschmidt and Wittmann (1970) as follows: (a) To avoid immobilization of proteins by free radical fixation to the sample gels, gelation of the sample solution was not performed. Instead, when preparing sample gels, "sample charging electrophoresis" was performed before the first dimension (1D) electrophoresis to charge proteins into gel pieces polymerized previously in the reduced conditions. (b) During electrophoresis, proteins migrate together with charged reductants; therefore, 2-aminoethanethiol was used to avoid the formation of artificial disulfide bridges during migration. (c) The second dimension (2D) electrophoresis was carried out at a more acidic pH (3.6) to achieve better separation of very small and basic proteins. These modifications improved the quantitative yield and reproducibility of the 2D PAGE analyses, and many faint spots disappeared not only at the high MW side but also in the region containing the primary spots of r-proteins. Sample charging electrophoresis was carried out at 100 constant volts (CV) for 15 min at room temperature (RT). Subsequently, 1D electrophoresis was carried out at 170 CV for 8 h at RT, and 2D electrophoresis was carried out at 100 CV for 15 h at RT. The 2D gels were stained with CBB G-250, and protein spots were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

4.9 | Protein identification by MALDI-TOF MS

The identification of proteins in the spots from the RFHR 2D PAGE gels were analyzed by Genomine Inc. (Gyeongsangbuk-do, Korea). The proteins were digested by trypsin, and a peptide mass fingerprinting analysis was performed using MALDI-TOF MS. The results were searched against the UniProt database using the Mascot Search engine. The genome sequence of *C. testosteroni* ATCC11996 was used for identification of the HPF homolog (*Ct*HPF-125; Accession No. EHN67804) and eight r-proteins (L10, L17, L19, L22, L27, L33, S11, and S17).

4.10 | Determination of the *Ct*HPF-125 and *Ct*HPF-119 copy numbers

Copy number refers to the molar ratio of a ribosome binding protein to a single 70S ribosomal particle, 30S subunit, or 50S subunit. The optical density (OD) of a protein spot on an RFHR 2D PAGE gel was determined by scanning the spot with a GS-800 calibrated densitometer. The molar amount of the protein was based on its OD value and MW (OD/MW). The OD/MW values for *Ct*HPF-125 and *Ct*HPF-119 were normalized against those for the r-proteins L9 and S6 or L14 and L17, respectively. These r-proteins were used as references because their protein copy numbers are known (Hardy, 1975; Tal et al., 1990; Wada, 1986a), and they were located near the spots of *Ct*HPF-125 and *Ct*HPF-119 in the RFHR 2D PAGE gels.

4.11 | Preparation of the *Ct*HPF-125, *Ct*HPF-119, *Ct*HPF-125(Δ106–125), and *Ct*HPF-125(Δ50–55) proteins

YB1035 = KRX (pTXB1-*Cthpf*-125), YB1036 = KRX (pTXB1-*Cthpf*-119), YB1037 = KRX [pTXB1-*Cthpf*-125 (Δ106–125)], and YB1038 = KRX [pTXB1-*Cthpf*-125 (Δ50–55)] were overexpressed by adding 0.2% rhamnose to the 802 medium. The cells were cultured for 7 h and collected. The PRS fractions were prepared. Each intein-tagged protein in the PRS fraction was purified using a column filled with Chitin beads (New England Biolabs, Inc.) and then cleaved by DTT to obtain the desired proteins, as described in the instruction manual for the IMPACT™ Kit (New England BioLabs Inc., New England). The purified proteins were dialyzed against Tris-KCl buffer (20 mM Tris–HCl (pH 7.6) and 30 mM KCl) for use in the in vitro translation and binding assays. The purified proteins were stored at −20°C until use.

4.12 | Detection of *Ct*HPF-119 by immunoblotting

Protein samples were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Immobilon-P transfer membrane; Merck Millipore, Burlington, MA, USA). Subsequently, the membranes were incubated with an anti-*Ct*HPF-119 (NCBI accession no. WP_003072415) polyclonal rabbit antibody against a specific sequence of 13 amino acids (KTRQQGPRATAKR), which was produced and purified by SCRUM Inc. (Tokyo, Japan). Anti-*Ct*HPF-119 reacted with *Ct*HPF-119 but did not react with *Ct*HPF-125. Anti-Rabbit IgG (Fc) Alkaline Phosphatase Conjugate (Promega, Madison, Wisconsin, USA) was used as a secondary antibody and detected using BCIP/NBT Color Development Substrate (Promega).

4.13 | Binding of *Ct*HPF-125 and *Ct*HPF-119 to ribosomes in vitro

HSRs were prepared from *C. testosteroni* ATCC11996 cells cultured for 1 day. The *Ct*-HSRs were mixed with *Ct*HPF-125 and/or *Ct*HPF-119 and then incubated at 30°C for 30 min. The mixtures were then layered onto a 30% sucrose cushion prepared in buffer I and centrifuged in a 90 Ti rotor (Beckman) at 155,000*g* for 4 h at 4°C. Each pellet was resuspended in buffer I and analyzed by RFHR 2D PAGE.

4.14 | Expression of *Ct*HPF-125 and *Ct*HPF-119 under heat and cold stress

C. testosteroni ATCC11996 cells were cultured at 30°C until logarithmic phase (Klett units: 50) and then incubated at 37°C for 1 h (heat shock) or at 20°C for 2 h (cold shock). The cells were then centrifuged and used to prepare the CR fractions. The CR proteins were analyzed by RFHR 2D PAGE and western blotting using an anti-*Ct*HPF-119 antibody.

4.15 | In vitro translation assay

In vitro transcription-translation protein synthesis (Shimizu et al., 2005) was performed using the PURExpress 1.0 kit (GeneFrontier, Chiba, Japan). The *E. coli* ribosomes in kit component Solution III were replaced with *C. testosteroni* ribosomes. *Ct*-ribosomes were prepared from logarithmic phase cells (Klett units: 50) or cells cultured for 1 day, using HiTrap Butyl FF columns (GE Healthcare Life Sciences, Chicago, IL, USA) (Shimizu et al., 2005; Shimizu & Ueda, 2010). To distinguish the in vitro synthesized protein from other proteins during SDS-PAGE, the DNA encoding *Ec*GST-(1–193) (theoretical MW: 22098.5) was amplified from the genomic DNA of *E. coli* W3110 using PCR primers 5′-*Ecgst*-F and 3′-*Ecgst* (1–193)-R (Table S1). Subsequently, a second amplification of the *Ecgst*-(1–193) DNA was performed using the first PCR product as the template and PCR primers 5′-T7pRQ-SD and 3′-*Ecgst* (1–193)-R (Table S1). The amplicon was purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and used as the template DNA in the transcription-translation reaction. The reactions were carried out for 2 h at 37°C in a 20 μL reaction mixture containing 1 μM ribosomes, 20 ng of template DNA, and 10 units of RNase Inhibitor (Super) (FUJIFILM-Wako, Osaka, Japan). The reaction mixtures were analyzed by SDS-PAGE using a Multi Gel II mini, 10%–20% linear gradient gel (Cosmo Bio Inc., Tokyo, Japan). After Coomassie brilliant blue staining, gel bands were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories) to measure the density of the synthesized protein.

4.16 | In vitro formation of 100S ribosomes from *C. testosteroni* ATCC11996 70S ribosomes by *Ec*RMF/*Ec*HPF or long HPF

The method of 100S ribosome formation in vitro followed the protocol described previously, the brief steps of which are summarized below.

a. HSRs were prepared from *C. testosteroni* cells cultured for 1 day and incubated with *Ec*RMF (10-fold) and/or *Ec*HPF (10-fold), or buffer I as a control, for 30 min at 37°C. The mixtures were then subjected to 5%–20% SDG centrifugation and divided into 18 SDG fractions. These fractions were precipitated with 10% TCA, separated on 16% Tricine SDS-PAGE gels, and analyzed by immunoblotting with an anti-*Ec*RMF or anti-*Ec*HPF antibody. The *Ec*RMF and *Ec*HPF proteins were purified as described in the previous study (Ueta et al., 2008).
b. CR fractions were prepared from *C. testosteroni* cells cultured for 1 day. The CR fractions were washed with high-salt buffer, dialyzed against dissociation buffer II [20 mM Tris–HCl (pH 7.6), 1 mM magnesium acetate, 30 mM ammonium acetate, and 6 mM 2-mercaptoethanol], and then separated into two aliquots. One aliquot was mixed with purified *Lp*HPF (1.5:1 molar ratio of *Lp*HPF to ribosomes), and the other was mixed with buffer I. The samples were incubated at 37°C for 30 min and then layered onto a 30% sucrose cushion prepared in buffer I and centrifuged in a 90 Ti rotor (Beckman) at 155,000*g* for 4 h at 4°C. Each pellet was resuspended in buffer I and subjected to 5%–20% SDG centrifugation and divided into 18 SDG fractions. Each sample was then precipitated with 10% TCA, separated on a 12% SDS-PAGE gel, and analyzed by immunoblotting with an anti-*Sa*HPF antibody, which reacted with *Lp*HPF. The *Lp*HPF protein was purified as described in the previous study (Ueta et al., 2013).

4.17 | Detection of *Lp*HPF, *Ec*RMF, and *Ec*HPF by immunoblotting

Protein samples were separated on 12% SDS-PAGE gels or 16% Tricine SDS-PAGE gels and then transferred to

PVDF membranes (Immobilon-P transfer membrane; Merck Millipore). The *Lp*HPF, *Ec*RMF, and *Ec*HPF proteins were detected with anti-*Sa*HPF, anti-*Ec*RMF, and anti-*Ec*HPF antibodies, respectively. Anti-Rabbit IgG (Fc) Alkaline Phosphatase Conjugate (Promega) was used as a secondary antibody and detected using BCIP/NBT Color Development Substrate (Promega).

AUTHOR CONTRIBUTIONS

M.U., A.W., and C.W. designed research (equal contribution); A.W. for RHFR method and M.U. for the other experiments performed; M.U., A.W., and C.W. analyzed data (equal contribution); C.W., M.U., and A.W. wrote the paper.

ACKNOWLEDGMENTS

We thank the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) of Japan for providing the *C. testosteroni* ATCC11996 (NBRC 14951^T) strain. We thank Kazuya Morikawa for providing pMADcm and pMADtet plasmid and Hideji Yoshida for technical support with Beckmann ultracentrifuge.

ORCID

Masami Ueta https://orcid.org/0000-0002-2858-0946
Akira Wada https://orcid.org/0000-0002-8810-8264
Chieko Wada https://orcid.org/0000-0003-2924-3594

REFERENCES

Agafonov, D. E., Kolb, V. A., Nazimov, I. V., & Spirin, A. S. (1999). A protein residing at the subunit interface of the bacterial ribosome. *Proceedings of the National Academy of Sciences of the United States of American*, *96*(22), 12345–12349. https://doi.org/10.1073/pnas.96.22.12345

Agafonov, D. E., Kolb, V. A., & Spirin, A. S. (2001). Ribosome-associated protein that inhibits translation at the aminoacyl-tRNA binding stage. *The European Molecular Biology Organization Report*, *2*, 399–402. https://doi.org/10.1093/embo-reports/kve091

Aiso, T., Yoshida, H., Wada, A., & Ohki, R. (2005). Modulation of mRNA stability participates in stationary-phase specific expression of ribosome modulation factor. *Journal of Bacteriology*, *187*, 1951–1958. https://doi.org/10.1128/JB.187.6.1951-1958.2005

Akiyama, T., Williamson, K. S., Schaefer, R., Pratt, S., Chang, C. B., & Franklin, M. J. (2017). Resuscitation of *Pseudomonas aeruginosa* from dormancy requires hibernation promoting factor (PA4463) for ribosome preservation. *Proceedings of the National Academy of Sciences of the United States of American*, *114*(2), 3204–3209. https://doi.org/10.1073/pnas.1700695114

Arnaud, M., Chastanet, A., & Debarbouille, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Applied and Environmental Microbiology*, *70*, 6887–6891. https://doi.org/10.1128/AEM.70.11.6887-6891.2004

Basu, A., & Yap, M. N. (2016). Ribosome hibernation factor promotes *Staphylococcal* survival and differentially represses translation. *Nucleic Acids Research*, *44*(10), 4881–4893. https://doi.org/10.1093/nar/gkw180

Battistuzzi, F. U., & Hedges, S. B. (2009). A major clade of prokaryotes with ancient adaptations to life and land. *Molecular Biology and Evolution*, *26*(2), 335–343. https://doi.org/10.1093/molbev/msn247

Beckert, B., Abdelshahid, M., Schäfer, H., Steinchen, W., Arenz, S., Berninghausen, O., ... Wilson, D. N. (2017). Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. *EMBO Journal*, *36*(14), 2061–2072. https://doi.org/10.15252/embj.201696189

Beckert, B., Turk, M., Czech, A., Berninghausen, O., Beckmann, R., Ignatova, Z., Plitzko, J. M., & Wilson, D. N. (2018). Structure of a hibernating 100S ribosome reveals an inactive conformation of the ribosomal protein S1. *Nature Microbiology*, *3*(10), 1115–1121. https://doi.org/10.1038/s41564-018-0237-0

Feaga, H. A., Kopylov, M., Kim, J. K., Jovanovic, M., & Dworkin, J. (2020). Ribosome dimerization protects the small subunit. *Journal of Bacteriology*, *202*(10), e00009-20. https://doi.org/10.1128/JB.00009-20

Flygaard, R. K., Boegholm, N., Yusupov, M., & Jenner, L. B. (2018). Cryo-EM structure of the hibernating *Thermus thermophilus* 100S ribosome reveals a protein-mediated dimerization mechanism. *Nature Communications*, *9*(1), 4179. https://doi.org/10.1038/s41467-018-06724-x

Franken, L. E., Oostergetel, G. T., Pijning, T., Puri, P., Arkhipova, V., Boekema, E. J., Poolman, B., & Guskov, A. (2017). A general mechanism of ribosome dimerization revealed by single-particle cryo-electron microscopy. *Nature Communications*, *8*(1), 722. https://doi.org/10.1038/s41467-017-00718-x

Fukuda, K., Hosoyama, A., Tsuchikane, K., Ohji, S., Yamazoe, A., Fujita, N., Shintani, M., & Kimbara, K. (2014). Complete genome sequence of polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 (NBRC 109938). *Genome Announcements*, *2*(5), e00865-14. https://doi.org/10.1128/genomeA.00865-14

Hardy, S. J. (1975). The stoichiometry of the ribosomal proteins of *Escherichia coli*. *Molecular and General Genetics*, *140*(3), 253–274. https://doi.org/10.1007/BF00334270

Hardy, S. J., Kurland, C. G., Voynow, P., & Mora, G. (1969). The ribosomal proteins of *Escherichia coli*. I. Purification of 30S ribosomal proteins. *Biochemistry*, *8*(7), 2897–2905. https://doi.org/10.1021/bi00835a03

Horie, K., Wada, A., & Fukutome, H. (1981). Conformational studies of *Escherichia coli* ribosomes with the use of acridine orange as a probe. *Journal of Biochemistry*, *90*(2), 449–461. https://doi.org/10.1093/oxforddjournals.jbchem.a133492

Horinouchi, M., Koshino, H., Malon, M., Hirota, H., & Hayashi, T. (2018). Steroid degradation in *Comamonas testosteroni* TA441: Identification of metabolites and the genes involved in the reactions necessary before D-ring cleavage. *Applied and Environmental Microbiology*, *84*(22), e01324-18. https://doi.org/10.1128/AEM.01324-18

Huang, W., & Wilks, A. (2017). A rapid seamless method for gene knockout in Pseudomonas aeruginosa. *BMC Microbiology*, *17*(1), 199. https://doi.org/10.1186/s12866-017-1112-5

Izutsu, K., Wada, A., & Wada, C. (2001). Expression of ribosome modulation factor (RMF) in *Escherichia coli* requires ppGpp. *Genes to Cells*, *6*(8), 665–676. https://doi.org/10.1046/j.1365-2443.2001.00457.x

Jones, S. E., Leong, V., Ortega, J., & Elliot, M. A. (2014). Development, antibiotic production, and ribosome assembly in *Streptomyces venezuelae* are impacted by RNase J and RNase III deletion. *Journal of Bacteriology*, *196*(24), 4253–4267. https://doi.org/10.1128/JB.02205-14

Kaltschmidt, E., & Wittmann, H. G. (1970). Ribosomal proteins, XII. Number of proteins in small and large ribosomal subunits of *Escherichia coli* as determined by two-dimensional gel electrophoresis. *Proceedings of the National Academy of Sciences of the United States of American*, *67*(3), 1276–1282. https://doi.org/10.1073/pnas.67.3.1276

Kato, T., Yoshida, H., Miyata, T., Maki, Y., Wada, A., & Namba, K. (2010). Structure of the 100S ribosome in the hibernation stage revealed by electron cryomicroscopy. *Structure*, *18*(6), 719–724. https://doi.org/10.1016/j.str.2010.02.017

Khusainov, I., Vicens, Q., Ayupov, R., Usachev, K., Myasnikov, A., Simonetti, A., Validov, S., Kieffer, B., Yusupova, G., Yusupov, M., & Hashem, Y. (2017). Structures and dynamics of hibernating ribosomes from *Staphylococcus aureus* mediated by intermolecular interactions of HPF. *EMBO Journal*, *36*(14), 2073–2087. https://doi.org/10.15252/embj.201696105

Kline, B. C., McKay, S. L., Tang, W. W., & Portnoy, D. A. (2015). *Listeria monocytogenes* hibernation-promoting factor is required for the formation of 100S ribosomes, optimal fitness, and pathogenesis. *Journal of Bacteriology*, *197*(3), 581–591. https://doi.org/10.1128/JB.02223-14

Lipońska, A., & Yap, M. F. (2021). Hibernation-promoting factor sequesters *Staphylococcus aureus* ribosomes to antagonize RNase R-mediated nucleolytic degradation. *American Society for Microbiology*, *12*(4), e00334-21. https://doi.org/10.1128/mBio.00334-21

Maki, Y., & Yoshida, H. (2021). Ribosomal hibernation-associated factors in *Escherichia coli*. *Microorganisms*, *10*(1), 33. https://doi.org/10.3390/microorganisms10010033

Maki, Y., Yoshida, H., & Wada, A. (2000). Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes to Cells*, *5*(12), 965–974. https://doi.org/10.1046/j.1365-2443.2000.00389.x

Matzov, D., Aibara, S., Basu, A., Zimmerman, E., Bashan, A., Yap, M.-N. F., Amunts, A., & Yonath, A. E. (2017). The cryo-EM structure of hibernating 100S ribosome dimer from pathogenic *Staphylococcus aureus*. *Nature communications.*, *8*(1), 723. https://doi.org/10.1038/s41467-017-00753-8

Noll, M., Hapke, B., Schreire, M. H., & Noll, H. (1973). Structural dynamics of bacterial ribosomes. I. Characterization of vacant couples and their relation to complexed ribosomes. *Journal of Molecular Biology*, *75*(2), 281–294. https://doi.org/10.1016/0022-2836(73)90021-1

Ortiz, J. O., Brandt, F., Matias, V. R. F., Sennels, L., Rappsilber, J., Scheres, S. H. W., Eibauer, M., Hartl, F. U., & Baumeister, W. (2010). Structure of hibernating ribosomes studied by cryo-electron tomography *in vitro* and *in situ*. *Journal of Cell Biology*, *190*(4), 613–4621. https://doi.org/10.1083/jcb.201005007

Polikanov, Y. S., Blaha, G. M., & Steitz, T. A. (2012). How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. *Science*, *336*(6083), 915–918. https://doi.org/10.1126/science.1218538

Prossliner, T., Gerdes, K., Sørensen, M. A., & Winther, K. S. (2021). Hibernation factors directly block ribonucleases from entering the ribosome in response to starvation. *Nucleic Acids Research*, *49*(4), 2226–2239. https://doi.org/10.1093/nar/gkab017

Puri, P., Eckhardt, T. H., Franken, L. E., Fusetti, F., Stuart, M. C., Boekema, E. J., Kuipers, O. P., Kok, J., & Poolman, B. (2014). *Lactococcus lactis* YfiA is necessary and sufficient for ribosome dimerization. *Molecular Microbiology*, *91*(2), 394–407. https://doi.org/10.1111/mmi.12468

Rak, A., Kalinin, A., Shcherbakov, D., & Bayer, P. (2002). Solution structure of the ribosome-associated cold shock response protein Yfia of *Escherichia coli*. *Biochemical Biophysical Research Communication*, *299*(5), 710–714. https://doi.org/10.1016/s0006-291x(02)02721-3

Sato, A., Watanabe, T., Maki, Y., Ueta, M., Yoshida, H., Ito, Y., Wada, A., & Mishima, M. (2009). Solution structure of the *E. coli* ribosome hibernation promoting factor HPF: Implications for the relationship between structure and function. *Molecular cell biology research communications*, *389*(4), 580–585. https://doi.org/10.1016/j.bbrc.2009.09.022

Sharma, M. R., Wilson, D. N., Datta, P. P., Schluenzen, F., Fucini, P., & Agrawal, R. K. (2007). Cryo-EM study of the spinach chloroplast ribosome reveals the structural and functional roles of plastid-specific ribosomal proteins. *Proceedings of the National Academy of Sciences of the United States of American*, *104*(49), 19315–19320. https://doi.org/10.1073/pnas.0709856104

Shimizu, Y., Kanamori, T., & Ueda, T. (2005). Protein synthesis by pure translation systems. *Methods*, *36*(3), 299–304. https://doi.org/10.1016/j.ymeth.2005.04.006

Shimizu, Y., & Ueda, T. (2010). PURE technology. *Methods in Molecular Biology*, *607*, 11–21. https://doi.org/10.1007/978-1-60327-331-2_2

Tagami, K., Nanamiya, H., Kazo, Y., Maehashi, M., Suzuki, S., Namba, E., Hoshiya, M., Hanai, R., Tozawa, Y., Morimoto, T., Ogasawara, N., Kageyama, Y., Ara, K., Ozaki, K., Yoshida, M., Kuroiwa, H., Kuroiwa, T., Ohashi, Y., & Kawamura, F. (2012). Expression of a small (p)ppGpp synthetase, YwaC, in the ppGpp mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome. *Microbiologyopen*, *1*(2), 115–134. https://doi.org/10.1002/mbo3.16

Tal, M., Weissman, I., & Silberstein, A. (1990). A new method for stoichiometric analysis of proteins in complex mixture–reevaluation of the stoichiometry of *E. coli* ribosomal proteins. *Journal of Biochemical and Biophysical Methods*, *21*(3), 247–266. https://doi.org/10.1016/0165-022x(90)90018-8

Tamaoka, J., Ha, D.-M., & Komagata, K. (1987). Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteron*i Marcus and Talaya 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. *International Journal of Systematic Bacteriology*, *37*(1), 52–59. https://doi.org/10.1099/00207713-37-1-52

Theng, S., Williamson, K. S., & Franklin, M. J. (2020). Role of hibernation promoting factor in ribosomal protein stability during *Pseudomonas aeruginosa* dormancy. *International Journal of Molecular Sciences*, *21*(24), 9494. https://doi.org/10.3390/ijms21249494

Tsai, M., Ohniwa, R. L., Kato, Y., Takeshita, S. L., Ohta, T., Saito, S., Hayashi, H., & Morikawa, K. (2011). *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiology*, *11*, 13. https://doi.org/10.1186/1471-2180-11-13

Ueta, M., Ohniwa, R. L., Yoshida, H., Maki, Y., Wada, C., & Wada, A. (2008). Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*. *Journal of Biochemistry*, *143*(3), 425–433. https://doi.org/10.1093/jb/mvm243

Ueta, M., Wada, C., Daifuku, T., Sako, Y., Bessho, Y., Kitamura, A., Ohniwa, R. L., Morikawa, K., Yoshida, H., Kato, T., Miyata, T., Namba, K., & Wada, A. (2013). Conservation of two distinct types of 100S ribosome in bacteria. *Genes to Cells*, *18*(7), 554–574. https://doi.org/10.1111/gtc.12057

Ueta, M., Wada, C., & Wada, A. (2010). Formation of 100S ribosomes in *Staphylococcus aureus* by the hibernation promoting factor homolog *Sa*HPF. *Genes to Cells*, *15*(1), 43–58. https://doi.org/10.1111/j.1365-2443.2009.01364.x

Ueta, M., Yoshida, H., Wada, C., Baba, T., Mori, H., & Wada, A. (2005). Ribosome binding proteins YfiA and YhbH have opposite functions during 100S formation in the stationary phase of *Escherichia coli*. *Genes to Cells*, *10*(12), 1103–1112. https://doi.org/10.1111/j.1365-2443.2005.00903.x

Usachev, K. S., Fatkhullin, B. F., Klochkova, E. A., Miftakhov, A. K., Golubev, A. A., Bikmullin, A. G., Nurullina, L. I., Garaeva, N. S., Islamov, D. R., Gabdulkhakov, A. G., Lekontseva, N. V., Tishchenko, S. V., Balobanov, V. A., Khusainov, I. S., Yusupov, M. M., & Validov, S. Z. (2020). Dimerization of long hibernation promoting factor from *Staphylococcus aureus*: Structural analysis and biochemical characterization. *Journal of Structural Biology*, *209*(1), 107408. https://doi.org/10.1016/j.jsb.2019.107408

Vila-Sanjurjo, A., Schuwirth, B., Hau, C. W., & Cate, J. H. D. (2004). Structural basis for the control of translational initiation during stress. *Nature Structural & Molecular Biology*, *11*(11), 1054–1059. https://doi.org/10.1038/nsmb850

Wada, A. (1986a). Analysis of *Escherichia coli* ribosomal proteins by an improved two-dimensional gel electrophoresis. I. Detection of four new proteins. *Journal of Biochemistry*, *100*(6), 1583–1594. https://doi.org/10.1093/oxfordjournals.jbchem.a121866

Wada, A. (1986b). Analysis of *Escherichia coli* ribosomal proteins by an improved two-dimensional gel electrophoresis. II. Characterization of four new proteins. *Journal of Biochemistry*, *100*(6), 1595–1605. https://doi.org/10.1093/oxfordjournals.jbchem.a121867

Wada, A. (1998). Growth phase coupled modulation of *Escherichia coli* ribosomes. *Genes to Cells*, *3*(4), 203–208. https://doi.org/10.1046/j.1365-2443.1998.00187.x

Wada, A., Yamazaki, Y., Fujita, N., & Ishihama, A. (1990). Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proceedings of the National Academy of Sciences of the United States of American*, *87*(7), 2657–2661. https://doi.org/10.1073/pnas.87.7.2657

Yamagishi, M., Matsushima, H., Wada, A., Sakagami, M., Fujita, N., & Ishihama, A. (1993). Regulation of *Escherichia coli rmf* gene encoding the ribosome modulation factor: Growth phase- and growth rate-dependent control. *EMBO Journal*, *12*(2), 625–630. https://doi.org/10.1002/j.1460-2075.1993.tb05695.x

Ye, K., Serganov, A., Hu, W., Garber, M., & Patel, D. J. (2002). Ribosome-associated factor Y adopts a fold resembling a double-stranded RNA binding domain scaffold. *European Journal of Biochemistry*, *269*(21), 5182–5191. https://doi.org/10.1046/j.1432-1033.2002.03222.x

Yoshida, H., Maki, Y., Kato, H., Fujisawa, H., Izutsu, K., Wada, C., & Wada, A. (2002). The ribosome modulation factor (RMF) binding site on the 100S ribosome of *Escherichia coli*. *The Journal of Biochemistry*, *132*(6), 983–989. https://doi.org/10.1093/oxfordjournals.jbchem.a003313

Yoshida, H., & Wada, A. (2014). The 100S ribosome: Ribosomal hibernation induced by stress. *Wiley Interdisciplinary Reviews: RNA*, *5*(5), 723–732. https://doi.org/10.1002/wrna.1242

Yoshida, H., Wada, A., Shimada, T., Maki, Y., & Ishihama, A. (2019). Coordinated regulation of Rsd and RMF for simultaneous hibernation of transcription apparatus and translation machinery in stationary-phase *Escherichia coli*. *Frontiers in Genetics*, *10*, 1153. https://doi.org/10.3389/fgene.2019.01153

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ueta, M., Wada, A., & Wada, C. (2024). The hibernation promoting factor of Betaproteobacteria *Comamonas testosteroni* cannot induce 100S ribosome formation but stabilizes 70S ribosomal particles. *Genes to Cells*, 1–22. https://doi.org/10.1111/gtc.13137