Ribosome dimerization is essential for the efficient regrowth of *Bacillus subtilis*

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Ribosome dimers are a translationally inactive form of ribosomes found in *Escherichia coli* and many other bacterial cells. In this study, we found that the 70S ribosomes of *Bacillus subtilis* dimerized during the early stationary phase and these dimers remained in the cytoplasm until regrowth was initiated. Ribosome dimerization during the stationary phase required the *hpf* gene, which encodes a homologue of the *E. coli* hibernation-promoting factor (Hpf). The expression of *hpf* was induced at an early stationary phase and its expression was observed throughout the rest of the experimental period, including the entire 6 h of the stationary phase. Ribosome dimerization followed the induction of *hpf* in WT cells, but the dimerization was impaired in cells harbouring a deletion in the *hpf* gene. Although the absence of ribosome dimerization in these Hpf-deficient cells did not affect their viability in the stationary phase, their ability to regrow from the stationary phase decreased. Thus, following the transfer of stationary-phase cells to fresh LB medium, Δ*hpf* mutant cells grew slower than WT cells. This observed lag in growth of Δ*hpf* cells was probably due to a delay in restoring their translational activity. During regrowth, the abundance of ribosome dimers in WT cells decreased with a concomitant increase in the abundance of 70S ribosomes and growth rate. These results suggest that the ribosome dimers, by providing 70S ribosomes to the cells, play an important role in facilitating rapid and efficient regrowth of cells under nutrient-rich conditions.

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INTRODUCTION

During entry into the stationary phase, the majority of 70S ribosomes in *Escherichia coli* dimerize to form 100S ribosomes (Wada et al., 1995; Yoshida et al., 2002). These 100S ribosomes are a translationally inactive form of ribosomes, the formation of which has been termed 'ribosomal hibernation' (Yoshida et al., 2002). Dimerization of 70S ribosomes requires the ribosome modulation factor (Rmf) and is promoted by the hibernation-promoting factor (Hpf, previously called YhbH), both of which are specifically expressed during the stationary phase (Maki et al., 2000; Ueta et al., 2005). When cells in the stationary phase are transferred to fresh medium, Rmf and Hpf are immediately released from the 100S ribosomes, as a result of which 100S ribosomes dissociate and form active 70S ribosomes (Aiso et al., 2005; Ueta et al., 2005).

In addition to *E. coli*, 100S ribosomes are also found in several gammaproteobacteria, such as *Salmonella typhimurium*, which harbour both Rmf and short Hpf (Ueta et al., 2013). In gammaproteobacteria, 100S ribosomes are detected in stationary-phase cells but not in exponential-phase cells (Ueta et al., 2013). Several other bacterial species not belonging to the gammaproteobacteria, such as *Staphylococcus aureus*, which harbour only long Hpf, also form 100S ribosomes (Ueta et al., 2010, 2013). In contrast to the gammaproteobacteria, ribosome dimers have been detected during both the exponential and

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Two supplementary figures and two supplementary tables are available with the online Supplementary Material.
stationary phases in several of these long-Hpf-harbouring bacteria (except Lactococcus lactis, Puri et al., 2014) when they are grown in nutrient-rich media (Ueta et al., 2010, 2013). Long Hpf has been found to induce dimerization of 70S ribosomes both in vitro and in vivo in Staph. aureus and Lactobacillus paracasei (Ueta et al., 2010, 2013).

Bacillus subtilis harbours a long Hpf homologue, which is encoded by the yvyD (herein designated hpf) gene, but has no Rmf homologue (Ueta et al., 2010; Tagami et al., 2012). The expression of the hpf gene in B. subtilis has been reported to be regulated by two alternative sigma factors, σ^H^ and σ^H^ (Drzewiecki et al., 1998). The σ^H^-dependent promoter mainly drives the expression of hpf under physical stress conditions, such as high salt, ethanol treatment and glucose starvation, whereas the σ^H^-dependent promoter has been shown to be activated under amino acid starvation conditions (Drzewiecki et al., 1998). B. subtilis harbours the relA gene, whose product is involved in the synthesis and/or hydrolysis of (p)ppGpp, and also has ybbM and ywaC genes, both of which encode small (p)ppGpp synthetases (Nanamiya et al., 2008). When the ywaC gene, under the control of an IPTG-inducible Pspac promoter and inserted at the aprE site of the (p)ppGpp^9^-mutant strain of B. subtilis (harbouring deletions in genes relA, ybbM and ywaC), is induced, hpf is transcribed exclusively from the σ^H^-dependent promoter and ribosome dimers are formed (Tagami et al., 2012). However, no ribosome dimerization is observed following the induction of ywaC in a B. subtilis strain harbouring either a σ^H^-null or hpf-null mutation, suggesting that ribosome dimer formation following the induction of the ywaC gene in the (p)ppGpp^9^-mutant is dependent on both Hpf and σ^H^ (Tagami et al., 2012).

The formation of ribosome dimers and their physiological significance in B. subtilis are still not fully understood. It is known that 100S ribosomes are essential for the survival of E. coli harbouring both Rmf and short Hpf in the stationary phase (Yamagishi et al., 1993; Wada et al., 2000). It is also known that Lc. lactis cells, which lack the long Hpf, do not form 100S ribosomes and exhibit defects in growth after these cells are starved for glucose for several days (Puri et al., 2014). Furthermore, a recent study has shown that Listeria monocytogenes cells require long Hpf, deficiency of which impaired 100S ribosome formation in these cells, for their competitive survival during prolonged cultivation when co-cultured with WT cells (Kline et al., 2015). Although the above studies suggest biological relevance of ribosome dimers, our understanding of the physiological impact of ribosome dimerization in bacteria and how this dimerization contributes to cell survival, e.g. under non-growing conditions, remains elusive.

In this study, we analysed the ribosome profiles of B. subtilis, grown in a nutrient-rich and non-sporulation medium, at different stages of growth and found that 70S ribosomes dimerized during the early stationary phase. The resulting 100S ribosomes were then stored until cell regrowth was initiated. The dimerization of 70S ribosomes correlated well with the expression of Hpf and ribosome dimerization was not detected in cells lacking this protein. Furthermore, in order to understand the physiological significance of ribosome dimerization in B. subtilis, we examined whether the absence of Hpf influenced the regrowth efficiency of stationary-phase cells and switching of ribosome dimers into 70S monomers.

**METHODS**

**Media and culture conditions.** LB (Sambrook & Russell, 2001) and LB agar were used for growing cells in liquid culture and on culture plates, respectively. In general, cells grown on LB agar plates for ~16 h at 28 °C were inoculated into LB medium, resulting in culture OD_{600} ~ 0.04, and then grown at 37 °C with shaking. For the regrowth analysis, cells grown on LB agar plates at 28 °C for ~16 h were used for inoculating 5 ml LB in L-shaped test tubes and grown for 24 h at 37 °C with shaking, the resulting culture was diluted with an appropriate volume of fresh LB medium to obtain final OD_{600} ~ 0.04, and the diluted culture was then incubated at 37 °C with shaking. When RIK211 [trpB'A' : P_{rrnO-kan} erm190(oc) hisC101] and RIK2540 [hpfD trpB'A' : P_{rrnO-kan}196(am) erm hisC101] cells were co-cultured for the competitive survival analysis, each strain was grown separately on LB agar plates at 28 °C for ~16 h, cells from each plate were used for inoculating LB medium to obtain separate cultures (OD_{600} of each culture ~ 0.04), and then an equal volume of each culture was mixed and the mixture was grown at 37 °C with shaking. Whenever necessary, the mixed culture was subcultured by diluting the culture with fresh LB to final OD_{600} 0.04. Culture conditions and media for preparing competent cells have been described previously (Ashikaga et al., 2000). When required, the following antibiotics were added to the growth medium at the concentrations specified: chloramphenicol (5 μg ml^{-1}), kanamycin (5 μg ml^{-1}) and erythromycin (0.5 μg ml^{-1}).

**Bacterial strains.** All B. subtilis strains used in the study were isogenic with B. subtilis strain 168 (trpC2). The RIK2508 strain (trpC2 Δhpf) was created as follows. Strain RIK1800 (hisC101), which was originally designated strain 168H (Nanamiya et al., 2010) but was renamed later by us as strain RIK1800 (Yano et al., 2013), was transformed with the chromosomal DNA of strain RIK1069 (trpC2 Δhpf : P_{rrnO-kan}) (Tagami et al., 2012) to obtain a kanamycin-resistant strain, RIK2506 (trpC2 Δhpf : P_{rrnO-kan} hisC101). A kanamycin-sensitive strain was derived from this strain as follows. First, two PCRs were performed using the chromosomal DNA of strain 168 as the template; in one PCR, primers Hpf-DUF and Hpf-DUR were used to amplify the upstream region of the hpf gene, whereas in the second PCR, primers Hpf-DUF and Hpf-DUR were used to amplify the downstream region of the hpf gene. Next, these two amplified fragments were simultaneously used as the template in another PCR amplification reaction with primers Hpf-DUF and Hpf-DUR to amplify DNA fragment 1. Another DNA fragment containing the hisC^+^ gene was amplified by primers TrpPCF and HisCR using the chromosomal DNA of strain 168 as the template. The resultant product was then mixed with fragment 1 and the mixture was used to transform strain RIK2506. Amongst the His^+^ transformants, a kanamycin-sensitive transformant was obtained and it was designated strain RIK2508 (trpC2 Δhpf). To detect the Δhpf mutant in a mixed culture, strain RIK2508 was transformed with chromosomal DNA of strain RIK213 [atrB'A' : P_{rrnO-kan}196(am) erm hisC101] (Nanamiya et al., 2010). Transformants were selected on the basis of their resistance to erythromycin and designated strain RIK2540 [hpfD ΔatrB'A' : P_{rrnO-kan}196(am) erm hisC101].
Strains RIK2601 (ΔtrpB’A’::Plac-yaaA-lacZ erm trpC2) and RIK2541 (Δhpf ΔtrpB’A’::Plac-yaaA-lacZ erm trpC2) were constructed as follows. To replace the cat gene of strain RIK1141 (ΔtrpB’A’::Pcat-cat erm181 trpC2) (Akanuma et al., 2006; Yano et al., 2013) with the lacZ gene, the upstream and downstream regions of the cat gene in the RIK1140 chromosome (ΔtrpB’A’::Pcat-cat erm trpC2) (Yano et al., 2013) were amplified using oligonucleotide primer combinations PrLacUF/PrLacUR and PrLacDF/PrLacDR, respectively. Next, the lacZ gene of RIK3106 (trpC2 anyf::Plac-yaaA-lacZ cat) (Suzuki et al., 2014) was amplified by PCR using the primers PrLacUF and PrLacLR. In another PCR, all three above-mentioned amplified fragments were used as the DNA template, and the PCR amplification reaction was carried out using the primers PrLacUF and PrLacLR. The resulting DNA fragment was then used to transform strain RIK2541, giving rise to the erythromycin-resistant transformant, RIK2601. To obtain the strain RIK2541, chromosomal DNA extracted from the RIK2601 strain was used to transform the strain RIK25208 and transformants exhibiting resistance to erythromycin were selected. Strains used in this study are listed in Table S1 (available in the online Supplementary Material) and oligonucleotide primers are listed in Table S2.

**Sucrose density gradient centrifugation.** Cells taken from LB plates were used for inoculating 400 ml LB medium in a 2 l Erlenmeyer flask and were grown at 37 °C with shaking (200 r.p.m.). Aliquots of cell culture were taken at the indicated times and cells from each aliquot were harvested by centrifugation. Culture volume used for the preparation of cell extract from each aliquot was determined such that the value of culture volume (ml) × OD600 was same. To prepare cell extracts, harvested cells were first resuspended in Buffer I (20 mM Tris/HCl, pH 7.6, 10 mM magnesium acetate, 100 mM ammonium acetate, 0.1 mM dithiothreitol, 2 mM PMSF) and the resuspended cells were disrupted by passing them through a French press (Aminco) at 55.2 MPa. Cell debris was removed by centrifugation as described previously (Natori et al., 2007; Tagami et al., 2012) and the supernatant was used as the crude cell extract. Whenever required, S100 pellet fractions were prepared as described previously (Suzuki et al., 2015). An aliquot of each sample (crude cell extract or S100 pellet fraction; 10 A260 units each) was layered onto a 10–40 % sucrose density gradient and the gradient was centrifuged at 65000 g in a Hitachi P40ST rotor for 17.5 h at 4 °C. After centrifugation, the gradient was fractionated using a Piston Gradient Fractionator (BioComp) and A234 profiles were monitored using a Bio-mini UV Monitor (ATTO).

**Preparation of an additional peak fraction containing ribosome dimers.** Aliquots of crude cell extract, prepared as described under ‘Sucrose density gradient centrifugation’, were centrifuged at 9500 g for 90 min at 4 °C in a Hitachi P55ST2 rotor. The resulting pellets were resuspended in 100 µl Buffer I and each resuspended pellet was layered onto a 10–40 % sucrose density gradient (100 A260 units per tube) and centrifuged at 4 °C for 16 h at 40000 g using a Hitachi P228S rotor, following which each gradient was fractionated. Fractions corresponding to the 70S ribosome peak and the 100S ribosome dimer peak were pooled separately. The pooled fractions were diluted with Buffer I (1 : 2 dilution) and ribosomes were recovered by centrifugation of each diluted fraction in a Hitachi P55ST2 rotor at 230 000 g for 3 h at 4 °C. The resulting ribosomal pellets were resuspended in 100 µl Buffer I and stored at −80 °C until use.

**Northern blot analysis.** Cells taken from LB plates were used for inoculating 400 ml LB in a 2 l Erlenmeyer flask and grown at 37 °C with shaking (200 r.p.m.). Aliquots were taken out at the indicated times and cells were harvested by centrifugation from each aliquot. Aliquot volume was adjusted such that the culture volume (ml) × OD600 = 15. Cell pellets were fast-frozen in liquid nitrogen and stored at −80 °C. For Northern blot analysis, total RNA was prepared from each cell pellet as follows. Each cell pellet was resuspended in 0.55 ml LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris/HCl, pH 7.4, 1 % SDS), to which 0.5 ml phenol/chloroform [50 % phenol, 49 % chloroform and 1 % isoamy alcohol (v/v)] and 0.5 g glass beads were added, and the mixture was vortexed vigorously. After addition of 4 ml LETS buffer and 2 ml phenol/chloroform, the mixture was vortexed again and then the aqueous phase was separated from the organic phase by centrifugation at 4 °C for 15 min at 5000 g. The aqueous phase (3.5 ml) was extracted with phenol/chloroform two more times, and nucleic acids were precipitated by the addition of 0.3 ml 4 M sodium acetate (pH 6.0) and 10 ml ethanol followed by centrifugation at 4 °C for 15 min at 18000 g. The pellet was washed with 70 % ethanol, briefly air-dried and then resuspended in 300 µl diethylpyrocarbonate-treated distilled water. Then, 900 µl 4 M sodium acetate was added to the solution and the mixture was kept at −20 °C for 16 h. Nucleic acids were pelleted by centrifugation at 4 °C for 15 min at 18000 g and the pellet was washed with 70 % ethanol. The pellet was resuspended with 300 µl DNease I buffer (40 mM Tris/ HCl, pH 7.5, 6 mM MgCl2) and treated with 2 U DNease I (Takara) for 30 min at 37 °C. After extracting with phenol/chloroform, RNA was precipitated by adding 15 µl 4 M LiCl and 900 µl ethanol, and the pellet was washed with 70 % ethanol. The pellet was resuspended in 200 µl diethylpyrocarbonate-treated distilled water and stored at −80 °C until use. Northern blot analysis of total RNA (15 µg) was performed as described previously using an hpf probe (Nanamiya et al., 2003; Tagami et al., 2012). The digoxigenin-labelled hpf probe was synthesized with a DIG RNA labelling kit (Roche Diagnostics). The template for the probe was amplified from the chromosomal DNA of B. subtilis 168 strain by using PCR primers hpf-PF and hpf-PR, which contained a recognition site for the T7 RNA polymerase. Following hybridization, the signal was amplified using ECF Substrate (GE Healthcare) and the amplified signal was detected using a Typhoon 9210 Variable Mode Imager (GE Healthcare).

**Western blot analysis.** Cells, which were taken from LB plates to inoculate 200 ml LB medium in 1 l Erlenmeyer flasks, were grown at 37 °C with shaking (200 r.p.m.) and aliquots of culture were removed at the indicated times. Cells were harvested from each aliquot [volume (ml) × OD600 = 5] by centrifugation and crude cell extracts were prepared from the harvested cells as described under ‘Sucrose density gradient centrifugation’. Crude cell extracts, each containing 20 µg total protein, or 0.1 A260 units of additional peak fractions, prepared as above, were electrophoresed on an SDS-polyacrylamide (12 %) minigel and the separated proteins were transferred to a PVDF membrane (Millipore). Procedures for Western blotting and detection of bound antibody were carried out as described previously (Nanamiya et al., 2003). Anti-Hpf antibody was prepared as described previously (Tagami et al., 2012) and used in the Western blot assay at a dilution of 1 : 1000. Anti-L2 antibody, prepared as described previously (Natori et al., 2007), was used at a dilution of 1 : 10 000. Anti-β-galactosidase antibody (Sigma) was used at a dilution of 1 : 1000.

**Primer extension analysis.** Total RNA was prepared from cells as described under ‘Northern blot analysis’. Total RNA (30 µg) was mixed with 1 pmol IRDye (infrared dye)-labelled oligonucleotide primer hpfP, the nucleotide sequence of which was complementary to the nucleotide sequence of the 5′-terminal region of the hpf gene, and a reverse transcription reaction was carried out using SuperScript III reverse transcriptase (Invitrogen) as described previously (Nanamiya et al., 2003, 2004; Natori et al., 2007). Products obtained from the reverse transcription reaction were separated on a 5 % polyacrylamide/6 M urea gel alongside a sequencing ladder, the template for which was amplified by PCR using primers hpf-TF and hpf-TR. Products obtained from the IRDye-labelled reverse transcription and DNA sequencing reactions were detected by using a LI-COR model 4300 DNA analyser (ALOKA).

**Sporulation assay.** Cells from LB plates were grown in 5 ml LB in an L-shaped test tube for 24 h at 37 °C with shaking. Viable cell count
was determined by plating aliquots of sequentially diluted cultures on LB agar plates. To determine the number of heat-resistant spores, cell cultures were heated at 80 °C for 10 min, aliquots were plated on LB agar and the plates were incubated at 37 °C for 24 h.

Quantification of rRNA. Total RNA was prepared from cells as described under ‘Northern blot analysis’. For the quantification of rRNA, the purified RNA was electrophoresed on a 1 % agarose gel. The applied volume was normalized using the formula: \( \frac{OD_{2\mu} \times CV_{2h}}{OD_{xh} \times CV_{xh}} \), where \( OD_{2\mu} \) is \( OD_{600} \) at 2 h after inoculation, \( OD_{xh} \) is \( OD_{600} \) at \( x \) h after inoculation, and \( CV_{2h} \) and \( CV_{xh} \) are culture volumes at 2 h and \( x \) h, respectively, after inoculation. Intensity of the band corresponding to 23S rRNA was determined using ImageJ software (http://imagej.nih.gov/ij/).

β-Galactosidase assay. Separate cultures (5 ml) of WT and \( \Delta hpf \) mutant cells harbouring \( Pr_{mtrc-lacZ} \) in L-shaped test tubes were grown for 24 h in LB medium at 37 °C with shaking and aliquots were used for inoculating fresh LB medium (50 ml each). Cells were grown at 37 °C with shaking (200 r.p.m.) and aliquots (0.5–4 ml) were collected at the indicated times. Whenever needed, chloramphenicol (100 μg ml\(^{-1}\)) was added to the culture 60 min after inoculation to inhibit translation. The β-galactosidase activity (Miller, 1972) was analysed as described previously, except that the samples were incubated at 28 °C (Nanamiya et al., 1998).

RESULTS

**B. subtilis** ribosomes dimerize during the early stationary phase

Recently we reported that the induction of the \( ywaC \) gene in a (p)ppGpp\(^{-} \) mutant of \( B. subtilis \), not a naturally occurring condition, resulted in the formation of ribosome dimers (Tagami et al., 2012). However, whether \( B. subtilis \) would form ribosome dimers under normal culture conditions is not known. To address this issue, we examined ribosome profiles of WT and \( \Delta hpf \) mutant cells grown in LB (a nutrient-rich and non-sporulation medium) at different stages of the growth cycle by sucrose density gradient sedimentation analysis. Fig. 1(a) shows the growth curves of \( B. subtilis \) WT and \( \Delta hpf \) mutant cells cultured in LB medium at 37 °C, and Fig. 1(b) shows the results of sedimentation analysis. In addition to the expected peaks of 30S, 50S and 70S ribosome particles, the sedimentation profiles also contained an additional peak (100S). In WT cells, the height of this additional peak increased significantly at the early stationary phase (3 h after inoculation) with a concomitant decrease in the height of the 70S peak and this additional peak was still observed at the late stationary phase (8 h after inoculation). We have previously detected a similar peak when \( YwaC \) was expressed in the (p)ppGpp\(^{-} \) mutant and have shown by electron microscopy studies that this peak contained dimers of 70S ribosomes (Tagami et al., 2012). We have also demonstrated that these ribosome dimers contained the Hpf protein and dimers were not formed in the absence of this protein (Tagami et al., 2012). Thus, based on the results obtained from the studied synthesis by Tagami et al. (2012), we suggest that this additional peak contains dimers of 70S ribosomes. However, the sedimentation profiles of \( \Delta hpf \) mutant cells also revealed an additional peak, the height of which did not increase at the early stationary phase (Fig. 1b). We believe that the additional peak that was observed in the sedimentation profiles of \( \Delta hpf \) mutant cells did not contain ribosome dimers, but may contain polysomes that would sediment on the sucrose gradient at an S-value similar to that of the ribosome dimers. Consistent with our previous observation (Tagami et al., 2012), Hpf was detected in the additional peak fraction that was prepared from WT cells at the stationary phase (3 h after inoculation), but not in the additional peak fraction that was prepared from the cells at the exponential phase (1 h after inoculation) (Fig. 1c). Moreover, the peak height of the additional peak fraction decreased when crude cell extract prepared from \( \Delta hpf \) mutant cells was first incubated at 37 °C for 120 min before the extract was subjected to density gradient centrifugation; this was, however, not the case when crude cell extract prepared from WT cells was incubated at 37 °C for 120 min before it was subjected to sucrose density centrifugation (Fig. S1). If the additional peak fraction mainly contained polysomes, which consist of active 70S ribosomes bound to mRNAs, the peak fraction would decrease as the 70S particles dissociate from the mRNAs under the above-described experimental conditions, as was demonstrated previously by Tagami et al. (2012). Taken together, these results strongly suggested that Hpf was required for the dimerization of 70S ribosomes in the stationary-phase \( B. subtilis \) cells.

To determine whether the expression pattern of Hpf paralleled ribosome dimerization, we next analysed hpf mRNA expression levels by Northern blot. As shown in Fig. 2(a), hpf mRNA was detected 2 h after inoculation (early stationary phase) and also throughout the entire experimental period (6 h) of the stationary phase, but not in the exponential phase (1 h after inoculation). It was shown previously that the expression of the hpf gene in \( B. subtilis \) is dependent on two minor sigma factors, \( \sigma^{B} \) and \( \sigma^{H} \) (Varón et al., 1996; Drzewiecki et al., 1998). When YwaC was expressed in the (p)ppGpp\(^{-} \) mutant, hpf was transcribed exclusively from the \( \sigma^{H} \)-dependent promoter (Tagami et al., 2012). To determine which sigma factor was responsible for the transcription of the hpf gene during the stationary phase, we performed a primer extension analysis. Active transcription of hpf was detected from both promoters in cells harvested at 2 h after inoculation (Fig. 2b). Furthermore, at 3 h after inoculation, transcription from the \( \sigma^{H} \)-dependent promoter was slightly higher than that from the \( \sigma^{B} \)-dependent promoter (Fig. 2b). Next, we monitored the expression level of Hpf protein by Western blot. As shown in Fig. 2(c), expression of Hpf was first detected at 3 h after inoculation and the expression level of Hpf protein remained constant at least up to 8 h after inoculation. Although a delay was observed between hpf mRNA expression and the appearance of Hpf protein, the expression of this protein correlated nicely with the initiation of ribosome dimerization (Fig. 1).
Therefore, it is most likely that the expression of hpf is an important factor that determines the formation of ribosome dimers in B. subtilis.

**Ribosome dimers are important for efficient regrowth**

Although one of the consequences of ribosome dimerization is translational repression, it was reported previously that the E. coli strain lacking the rmf gene, which did not form 100S ribosomes, is unable to survive for prolonged periods in the stationary phase (Yamagishi et al., 1993; Wada et al., 2000). Therefore, we next examined whether ribosome dimerization contributes to the survival of B. subtilis in the stationary phase. However, a comparative analysis of cell viability using the WT and Δhpf mutant (which is unable to form ribosome dimers) failed to reveal any significant difference, even at 120 h after inoculation (Fig. S2a). It should be noted that the sporulation efficiency of Δhpf mutant cells was almost the same as the sporulation efficiency of WT cells (Fig. S2b). Although no difference in cell growth and cell viability was observed when WT and Δhpf mutant cells were grown separately (Fig. S2a), prolonged cultivation of mixed cultures of WT and Δhpf mutant cells revealed that the viability of Δhpf mutant cells decreased more rapidly than that of WT cells (Fig. 3a). In E. coli, 100S ribosomes dissociate into active 70S ribosomes within 2 min of transferring the stationary-phase cells to a nutrition-rich culture medium (Wada, 1998). This previous observation in E. coli led us to suggest that the ribosome dimers formed in B. subtilis at the stationary phase might dissociate rapidly during regrowth to produce functionally active 70S ribosomes, and consequently increase the efficiency of

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**Fig. 1.** Growth curves and ribosome sedimentation profiles of WT and Δhpf mutant B. subtilis. (a) For growth curves, WT (filled circles) and Δhpf mutant (open triangles) cells were grown in LB. Results show mean ± SD of three independent experiments. (b) For sedimentation analysis, cells were grown as described in Methods and aliquots of the culture were removed at the indicated times. Cell extracts were prepared from cells harvested from each aliquot [aliquot volume (ml) × OD<sub>600</sub> = 100]. Each cell extract (10 A<sub>260</sub> units each) was sedimented through a 10–40% sucrose gradient as described in Methods. The 30S, 50S and 70S peaks are indicated in the top profile. An additional peak, which appeared in the sedimentation profiles of pre- and post-exponential-growth-phase cells and contained ribosome dimers and polysomes, is marked using a dashed line. (c) Detection of Hpf in the additional peak fraction by Western blot. Additional peak fractions were prepared from cells grown as above and harvested at exponential growth phase (1 h) and at 3 h. Peak fractions (0.1 A<sub>260</sub> units each) were subjected to Western blot analysis using the anti-Hpf antibody as described in Methods. Ribosomal protein L2 was used as a control and detected by Western blot using an anti-L2 antibody (Natori et al., 2007). For this blot, two sets of each sample were loaded onto the SDS-PAGE gel. After the gel electrophoresis and transfer of proteins to the membrane, the membrane blot was cut into two halves (each containing the indicated samples), one of which was probed with the anti-Hpf antibody and the other was probed with the anti-L2 antibody.
cell proliferation. To test this possibility, we inoculated the Δhpf mutant and WT cells, grown for 24 h in LB medium, into fresh medium and observed their growth characteristics. As shown in Fig. 3(b), regrowth of stationary-phase Δhpf mutant cells was significantly delayed compared with that of stationary-phase WT cells: the doubling time of WT cells during the lag phase (until 100 min after inoculation) was 57.5 ± 2.7 min, whereas that of Δhpf mutant cells was 149 ± 13 min. In contrast, when cells from the exponential growth phase were used as inocula, growth of these two cell types was virtually identical (Fig. 3b): doubling times of WT and Δhpf mutant cells were 17.7 ± 0.4 and 17.9 ± 0.2 min, respectively, indicating that when cells from the exponential growth phase were used as inocula, the growth of these two cell types was virtually identical. Although Δhpf mutant cells displayed a long lag phase during regrowth, the final OD_{600} of the Δhpf mutant cell culture reached the same value as the final OD_{600} of the WT cell culture (results not shown). To further elucidate the advantages of ribosome dimer formation, relative survival rates of WT and Δhpf mutant cells grown in a mixed culture and repeatedly subcultured were determined. Accordingly, both WT and Δhpf mutant cells were grown together in the LB medium until the culture reached the stationary phase (8 h growth), the culture was then appropriately diluted every 24 h with fresh LB medium, and the subculture process was repeated several times. As expected, the abundance of Δhpf mutant cells decreased compared with that of WT cells at each subculture step (Fig. 3c). These results suggested that, as far as regrowth from the stationary phase is concerned, the ribosome-dimer-forming WT cells had advantages over Δhpf mutant cells.

A previous study showed that the 70S ribosomes are more resistant to degradation by RNA hydrolases than the 30S and 50S subunits (Zundel et al., 2009). This observation raised the possibility that the ribosome dimers, which consist of 70S complexes, are involved in the stabilization of ribosomes in the stationary-phase cells. Therefore, we assumed that the observed decrease in the efficiency of regrowth of the Δhpf mutant was caused by a lower abundance of intracellular ribosomes in the late stationary phase. To explore this possibility, we compared changes in the ribosome content of WT and Δhpf mutant cells until they reached the late stationary phase. Although the abundance of ribosomes, which was measured by quantifying the amount of 23S rRNA, was reduced in both WT and Δhpf mutant cells in the stationary phase, the abundance of ribosomes in Δhpf mutant cells was significantly lower than that in WT cells (Fig. 4). This result suggested that ribosome dimerization was important for maintaining the intracellular abundance of ribosomes in the stationary phase.

Decrease in ribosome dimers during regrowth leads to a concomitant increase in 70S ribosomes

To determine whether the observed difference between the regrowth efficiency of WT and Δhpf mutant cells from the late stationary phase was caused by a disparity in the translational activity of the cells that were beginning to proliferate, we analysed changes in lacZ reporter gene translation by determining the β-galactosidase activity as well as LacZ protein content. For this purpose, we created WT and Δhpf mutant strains harbouring the E. coli lacZ gene under the control of strong and constitutive P1 and P2 promoters of the rrnO operon (P_{rrnO}), designated RIK2601 and RIK2541, respectively. We then used these strains to perform β-galactosidase and Western blot assays, postulating that the expression of β-galactosidase would depend on the cell’s translational activity. When late-stationary-phase cells were used for inoculating fresh LB medium, the β-galactosidase activity in RIK2601 cells increased more rapidly than that of RIK2541 cells (Fig. 5a). The expression levels of LacZ protein found in these cells (Fig. 5b) correlated nicely with their β-galactosidase activities (Fig. 5a). When chloramphenicol was added to the culture media of these cells, the observed increase in both β-galactosidase activity and LacZ expression level during regrowth was inhibited (Fig. 5a, b). As the transcriptional activity of P_{rrnO} could influence the outcome of this assay, we repeated the experiment using WT and Δhpf mutant cells harbouring the lacZ gene under the control of the IPTG-inducible P_{spac} promoter. In this case too, we observed that the β-galactosidase activity in WT cells
increased more rapidly with time than that in Δhpf mutant cells (results not shown). Taken together, these results suggested that the translational activity of stationary-phase WT cells, which contained ribosome dimers, appeared to recover faster than that of stationary-phase Δhpf mutant cells, which lacked ribosome dimers, at least in the context with the translation of the lacZ reporter gene. Additional experiments, such as analysis of

³⁵S-labelled methionine incorporation into proteins, will be needed to fully understand the underlying reasons for the restoration of translational activity in stationary-phase cells during their regrowth.

As restoration of growth as well as translation of the lacZ reporter gene was more rapid during the regrowth of stationary-phase WT cells than stationary-phase Δhpf

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**Fig. 3.** *hpf* is required for the efficient regrowth of cells from the stationary phase. (a) Cultures of WT cells harbouring the kanamycin resistance (*kan*) gene (RIK211) and Δhpf mutant cells harbouring the erythromycin resistance (*erm*) gene (RIK2540) were inoculated together into LB medium, and the mixed culture was incubated at 37 °C with shaking as described in Methods. Viable cell count was determined by plating appropriately diluted aliquots of the mixed culture, taken out at the times indicated, on LB agar plates containing either kanamycin (for the WT; open circles) or erythromycin (for the Δhpf mutant; open triangles). (b) WT (circles) and Δhpf mutant (triangles) cells, which were grown in LB medium at 37 °C with shaking for 40 min (filled symbols) or for 24 h (open symbols), were used for inoculating fresh LB medium. Cells were then incubated at 37 °C and their growth was monitored by measuring OD₆₀₀ at various times after inoculation. (c) Mixed cultures of WT cells harbouring the *kan* gene and Δhpf mutant cells harbouring the *erm* gene, prepared as described in (a), were incubated at 37 °C with shaking. The culture was diluted every 24 h with fresh LB medium to OD₆₀₀ 0.04 and regrown. For each strain, the viable cell count was determined by plating appropriately diluted aliquots on LB agar plates containing either kanamycin (for WT) or erythromycin (for Δhpf mutant) at the times indicated (up to 8 h following each subculture step). Open circles, WT; open triangles, Δhpf mutant. Results show mean ± SD of three independent experiments.
OD 600 of the culture and culture volume as described in Methods. The sample volume applied per lane was normalized with respect to the 23S rRNA band in each lane. Results show mean ± SD of relative intensities from three independent experiments.

**Fig. 4.** *hpf* is required for maintaining the ribosome content in cells in the late stationary phase. Total RNAs extracted from cells grown in LB at the indicated times [culture volume (ml) × OD<sub>600</sub> = 15] were electrophoresed on 1 % agarose gel. The sample volume applied per lane was normalized with respect to OD<sub>600</sub> of the culture and culture volume as described in Methods. Ribosome content was determined from the relative intensity of the 23S rRNA band in each lane. Results show mean ± SD of relative intensities from three independent experiments.

Fig. 4. *hpf* is required for maintaining the ribosome content in cells in the late stationary phase. Total RNAs extracted from cells grown in LB at the indicated times [culture volume (ml) × OD<sub>600</sub> = 15] were electrophoresed on 1 % agarose gel. The sample volume applied per lane was normalized with respect to OD<sub>600</sub> of the culture and culture volume as described in Methods. Ribosome content was determined from the relative intensity of the 23S rRNA band in each lane. Results show mean ± SD of relative intensities from three independent experiments.

mutant cells, we assumed that the recovery of translational activity in WT cells was likely due to the dissociation of dimers into functionally active 70S ribosomes. We therefore analysed the ribosome profiles of both WT and Δ*hpf* mutant cells during regrowth. In the case of WT cells, ribosome dimers were consistently observed when the stationary-phase cells were grown in LB medium for 24 h (Fig. 5c). Furthermore, the ribosome-dimer-containing peak decreased, and the 70S ribosome- and polysome-containing peaks concomitantly increased with the regrowth time (Fig. 5c). We confirmed that the level of Hpf in these cells also decreased as the level of ribosome dimers declined (Fig. 5d). These results suggested that ribosome dimers, which are formed early in the stationary phase of WT cells, dissociate to provide active 70S ribosomes during their regrowth. In contrast, the polysome-containing peak, which contained ribosomes actively translating mRNAs, was detected for at least 150 min after the regrowth of the Δ*hpf* mutant cells was initiated, with only a gradual increase in the 70S ribosome peak (Fig. 5c). This result suggested that there were lower numbers of active 70S ribosomes in *hpf* mutant cells than in WT cells, and that this result was in good agreement with the result obtained from the lacZ translation assay (Fig. 5a, b).

**DISCUSSION**

In the present study, we demonstrated that ribosome dimers are formed in *B. subtilis* early in the stationary phase and that their formation, which is dependent on Hpf, provides a mechanism for stabilizing ribosomes during a prolonged stationary phase. Our results confirmed that the long Hpf of *B. subtilis*, similar to the long Hpfs of other bacteria, plays a central role in ribosome dimerization. Although ribosome dimers are required for the survival of *E. coli* cells during the stationary phase, we found that the absence of Hpf had no influence on the viability of *B. subtilis* cells during the stationary phase when Δ*hpf* mutant cells were grown by themselves (Fig. 5a). However, the viability of Δ*hpf* mutant cells in mixed culture with WT cells decreased upon prolonged growth (Fig. 5a). This result is in agreement with the results of Kline *et al.* (2015), where it was shown that Hpf-lacking *Lt. monocytogenes* cells displayed a competitive survival disadvantage when co-cultured with WT cells. Moreover, it appears that ribosome dimerization is required for the efficient regrowth of stationary-phase cells. Thus, the Δ*hpf* mutant, which does not form ribosome dimers, exhibits a longer lag phase than the WT (Fig. 3b), presumably because of the delayed activation of the translational activity in the mutant (Fig. 5a, b). Accordingly, during regrowth, the observed increase in translation of the lacZ reporter preceded an increase in the growth rate (Figs 3b and 5a). In this regard, it should be noted that > 50 % of the total energy produced in bacteria is consumed during ribosome biogenesis and ribosomes account for as much as 50 % of the dry cell mass of bacteria (Nierhaus, 2004). Bacterial cells synthesize proteins more actively in the exponential phase than in the stationary phase; the number of ribosomes in a slowly growing cell is only 2000, whereas in a rapidly growing cell the number can be as high as 70000 (Kaczanowska & Rydén-Aulin, 2007).

In the present study, we also observed that the abundance of ribosomes in both WT and Δ*hpf* mutant cells decreased during the stationary phase. However, the amount of ribosomes retained in Δ*hpf* mutant cells during the stationary phase was less than that in stationary-phase WT cells (Fig. 4). As 70S ribosomes are more resistant to degradation by RNA hydrolases than 30S and 50S subunits (Zundel *et al.*, 2009), ribosome dimerization, which holds the 70S complex, is effective in stably storing the ribosomes during the stationary phase. The observed decrease in the ribosome dimer peak with a concomitant increase in the 70S ribosome peak (Fig. 5c) during the transition of WT cells from the stationary phase to the exponential phase indicates that the ribosome dimers, stored during the stationary phase, dissociate into active 70S ribosomes. This would account for their efficient regrowth upon subculture. Low abundance of ribosomes in the late stationary phase of the Δ*hpf* mutant was probably due to the absence of ribosome dimers. This result suggested that additional 70S ribosomes need to be synthesized before the regrowth of the Δ*hpf* mutant can be initiated. The energy required for this synthesis is likely responsible for the long lag phase that was observed upon subculture. However, it may be possible that most of the 70S ribosomes in the stationary-phase cells of both WT and Δ*hpf* mutant cells...
are not translationally competent, and they are activated by an unknown mechanism during regrowth. Consistent with this idea, both WT and Δhpf mutant cells displayed a lag phase in growth and an increase in translational activity. However, the mechanism for ribosome activation during regrowth remains unclear. Dimerization of ribosomes, therefore, fulfils a number of roles as illustrated in Fig. 6: (1) dimerized ribosomes are less susceptible to degradation by RNases, as a consequence of which their abundance increases during the extended stationary phase; (2) ribosome dimers are translationally inactive and therefore they do not add to the cell’s metabolic burden under low nutrient conditions; and (3) monomerization of ribosome dimers, triggered by the addition of nutrients, rapidly provides the means to enhance the rate of protein synthesis and improve the competitiveness of the bacterium. A long lag phase could impair the survivability of cells, particularly in the presence of competing cells. Consistent with this idea, an experiment performed using mixed cultures of WT and Δhpf mutant cells revealed that the number of

**Fig. 5.** Translation of the lacZ reporter is increased during regrowth. WT and Δhpf mutant cells harbouring P_{rrnO-lacZ} were grown for 24 h at 37 °C, aliquots were used for inoculating fresh LB medium, and cells were incubated at 37 °C with shaking. (a) Aliquots (0.5–4 ml) were collected at the indicated times and assayed for β-galactosidase activity as described in Methods. Results show mean±SD of three independent experiments. (b) Expression levels of LacZ protein in cell extracts (20 μg protein) prepared from cells harvested at the indicated times were detected by Western blotting with the anti-LacZ antibody as described in Methods. (c, d) Changes in the ribosome sedimentation profiles (c) and Hpf levels (d) of WT and Δhpf mutant cells during regrowth. WT and Δhpf mutant cells were grown separately in LB medium at 37 °C with shaking for 24 h, and aliquots from each were used to inoculate fresh LB media. Cell cultures were then incubated at 37 °C and cells were harvested at the indicated times. (c) The S100 pellet fraction (10 A_{260} units each) prepared from each culture [culture volume (ml) × OD_{600}=50] was sedimented through a 10–40 % sucrose gradient. Ribosome-dimer-containing peaks are indicated using dashed lines and polysome-containing peaks are indicated using arrows. (d) Hpf protein level in WT cells at various times following inoculation was detected by Western blot using an anti-Hpf antibody. Cell extract in each lane contained 20 μg protein.
viable mutant cells decreased very dramatically, compared with that of WT cells, after several rounds of subculture (Fig. 3c). One possible reason why Hpf is required for maintaining the viability of cells in the late stationary phase only under competitive conditions (Fig. 3a) is that the regrowth rate of Δhpf mutant cells in the stationary phase, as compared with that of WT cells, is decreased when the room for cell proliferation is created as a result of lysis of a portion of cells during the stationary phase. In this regard it should be noted that *B. subtilis* lyse their own sister cells under nutritional stress conditions to survive on the nutrients released from the lysed cells (González-Pastor et al., 2003; Nandy & Venkatesh, 2008). Whatever the reason behind this decrease in viability in cells lacking the *hpf* gene, it appears that the *hfp* gene has been conserved amongst prokaryotes because it provides a survival advantage under competitive growth conditions.

As discussed above, dimerization of ribosomes in the stationary phase is important for the efficient regrowth of cells; dimerized ribosomes are stored by the stationary-phase cells; when these stationary-phase cells are allowed to regrow in fresh medium, the stored dimers dissociate to form active 70S ribosomes and provide them to the growing cells. The peak corresponding to the ribosome dimer in the sedimentation profile and Hpf protein level in WT cells remained almost unchanged for at least 30 min after the cells began to regrow (Fig. 5c, d), suggesting that the dissociation of ribosome dimers of *B. subtilis* was slow compared with the dissociation of the *E. coli* 100S ribosome, which has been shown to dissociate completely within 2 min (Wada, 1998). These results are in good agreement with the observation that ribosome dimers formed by the long Hpf in *Lb. paracasei* were more stable than those formed by Rmf and short Hpf of *E. coli* (Ueta et al., 2013). Further investigation is needed to reveal the underlying mechanisms involved in the dissociation of ribosome dimers in *B. subtilis* during regrowth.

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**Fig. 6.** Model depicting the utilization cycles of ribosomes in *B. subtilis*. Ribosomes are dimerized at early exponential phase and their formation is dependent on Hpf. Dimerized ribosomes are stored until cell regrowth is initiated. When regrowth is triggered by the addition of nutrients, ribosome dimers dissociate to form active 70S ribosomes by unknown mechanisms and provide the means to enhance the rate of protein synthesis. In Δhpf mutant cells, ribosomes cannot form dimers and are probably degraded during the extended stationary phase. Thus, additional 70S ribosomes must be synthesized prior to regrowth. The energy required for the synthesis of additional ribosomes is likely responsible for the long lag phase that is observed upon subculture. See text for details.
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