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Lactococcus lactis YfiA is necessary and sufficient for ribosome dimerization

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Summary

Dimerization and inactivation of ribosomes in *Escherichia coli* is a two-step process that involves the binding of ribosome modulation factor (RMF) and hibernation promotion factor (HPF). *Lactococcus lactis* MG1363 expresses a protein, YfiA^{LI}, which associates with ribosomes in the stationary phase of growth and is responsible for dimerization of ribosomes. We show that full-length YfiA^{LI} is necessary and sufficient for ribosome dimerization in *L. lactis* but also functions heterologously *in vitro* with *E. coli* ribosomes. Deletion of the *yfiA* gene has no effect on the growth rate but diminishes the survival of *L. lactis* under energy-starving conditions. The N-terminal domain of YfiA^{LI} is homologous to HPF from *E. coli*, whereas the C-terminal domain has no counterpart in *E. coli*. By assembling ribosome dimers *in vitro*, we could dissect the roles of the N- and C-terminal domains of YfiA^{LI}. It is concluded that the dimerization and inactivation of ribosomes in *L. lactis* and *E. coli* differ in several cellular and molecular aspects. In addition, two-dimensional maps of dimeric ribosomes from *L. lactis* obtained by single particle electron microscopy show a marked structural difference in monomer association in comparison to the ribosome dimers in *E. coli*.

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Introduction

Ribosomes are ribonucleoprotein complexes that catalyse the synthesis of proteins in all organisms. In bacteria a functional ribosome has a sedimentation coefficient of 70S and is formed from the assembly of one small 30S and one large 50S subunit. The smaller 30S subunit has an approximate molecular weight of 0.9 MDa and consists of a 16S rRNA molecule and 21 ribosomal proteins annotated as S1-S21. The larger 50S subunit has an approximate molecular weight of 1.8 MDa and consists of two rRNA molecules (23S and 5S) and 33 ribosomal proteins annotated as L1-L36 (Kato *et al.*, 2010; Jenner *et al.*, 2012). During logarithmic growth of bacteria, the majorities of the ribosomes are involved in translation and are arranged as 70S complexes on mRNA to form a polysome chain similar to beads on a string. As soon as the translation process is completed the two subunits are separated, they are recycled and come together upon binding with another mRNA molecule. During the transition to stationary phase, when protein synthesis activity slows down due to nutrient starvation or stressful conditions, *E. coli* 70S ribosomes can form 90S dimers upon binding with a small 6.5 kDa protein, ribosome modulation factor RMF (Yamagishi *et al.*, 1993; Izutsu *et al.*, 2001). These intermediate ribosome dimers can subsequently bind a hibernation promotion factor (the 10.8 kDa protein, HPF) molecule to form a mature 100S ribosomal particle, in which the dimerization interface is made by the two 30S subunits of the two participating ribosomes (Kato *et al.*, 2010).

Dimerization of ribosomes in *E. coli* is a stationary phase process that increases the viability of the cell (Yamagishi *et al.*, 1993). The ribosome dimers represent a hibernation state and are translationally inactive (Wada *et al.*, 1995). Ribosomal hibernation has been suggested to constitute a method for cells to prevent ribosomes from being degraded by ribonucleases (Zundel *et al.*, 2009). A third protein that can bind to ribosomes when *E. coli* cells enter the stationary phase is YfiA (previously known as RaiA; Agafonov *et al.*, 1999). HPF and YfiA are structurally similar, and by X-ray diffraction and cryo-electron tomography studies it was demonstrated that both proteins can bind to the catalytic A- and P-sites of the ribosome (Vila-Sanjurjo *et al.*, 2004; Ortiz *et al.*, 2010). By creating heterologous complexes of *Thermus thermophilus* ribosomes with *E. coli*

YfiA, HPF, RMF and combination of these proteins, detailed information was obtained on their interactions with the ribosome and a mechanism of ribosome inactivation was proposed (Polikanov *et al*., 2012). RMF blocks ribosome binding to mRNA by preventing interaction of the messenger with 16S rRNA (Polikanov *et al*., 2012). HPF and YfiA have nearly identical binding sites in the ribosome, which overlap the sites where mRNA, tRNA and initiation factors would normally bind. Both HPF and YfiA are in the immediate vicinity of ribosomal proteins S9 and S10 (Kato *et al*., 2010). Upon binding of HPF and RMF to the ribosome, their positively charged surfaces are close to conserved residues in the ribosomal peptidyl transferase centre, which is proposed to inhibit the translation process. When bound to the ribosomes the C-terminal tail of *E. coli* YfiA interferes with the binding of RMF, thus preventing dimerization and resulting in the formation of translationally inactive monomeric 70S ribosomes (Ueta *et al*., 2005; Polikanov *et al*., 2012).

Multiple sequence alignments and phylogenetic analyses indicate that most bacteria have at least one HPF homologue. These homologues have been classified into three types: long HPF, short HPF and YfiA, on the basis of presence of a conserved domain and additional homologous sequences (Ueta *et al*., 2008). An open reading frame annotated as *yfiA* occurs in the genome of the lactic acid bacterium *Lactococcus lactis* strain MG1363. The encoded protein $YfiA^{Ll}$ belongs to the long HPF type. It shares 32% amino acid sequence identity with *E. coli* $YfiA^{Ec}$ and 64% identity with HPF from *Staphylococcus aureus* (Ueta *et al*., 2008). The *L. lactis* genome does not contain orthologues of *rmf* and *hpf* (Wegmann *et al*., 2007). A similar make up has been recently reported for *S. aureus*, five Lactobacillaceae species, *T. thermophilus* and *Synechocystis*, which all carry a gene coding for a long HPF but not for RMF or for short HPF (Ueta *et al*., 2013). In the same publication it was shown that the *Lactobacillus paracasei* long HPF protein caused a 65% inhibition of protein synthesis in an *in vitro* transcription-translation assay (Ueta *et al*., 2013).

In *E. coli* the number of ribosomes increases proportionally with the rate of growth (Gausing, 1974; Scott *et al*., 2010), indicating that protein synthesis is tuned to cellular needs. On the contrary, the number of ribosomes in *L. lactis* increases less than proportionally, which indicates that at a low growth rate the cell has excess protein synthesis capacity (A. Goel *et al*., manuscript in preparation). To prevent wasteful protein synthesis, cells need to control the activity of existing ribosomes. We thus searched for a protein factor that would inactivate a fraction of the ribosomes when *L. lactis* cells are growing at a low rate or entering the stationary phase. We performed a proteomic screen and found $YfiA^{Ll}$ to be associated with 100S ribosomes, the dominant ribosomal species in stationary phase-grown *L. lactis* cells. We analysed the domain structure of $YfiA^{Ll}$ and found that full-length lactococcal YfiA is essential for the formation of ribosomal dimers.

Results

100S ribosomes and identification of YfiA in L. lactis

Ribosomes were isolated from *L. lactis* cells in the exponential and stationary phases of growth and analysed using sucrose density gradient centrifugation. Ribosomes from exponentially growing cells gave a peak at 70S (Fig. 1A), which by negative-stain electron microscopy (EM) was shown to contain monomeric ribosomes (Fig. 1B). On the contrary, ribosomes isolated from stationary phase-growing cells sedimented as significantly larger particles, predominantly as 100S species (Fig. 1A and C). EM images of ribosome particles from exponential and stationary phase grown cells were analysed through single particle averaging (Fig. 1D1–D5). The dimeric ribosomes isolated from the stationary phase cells were observed in one dominant type of projection (Fig. 1D1). This map reveals a quasi twofold rotational symmetry, indicating that the monomers are seen in the same position, but rotated over 180° in the plane. By comparison with known high-resolution structures (Kato *et al*., 2010) it can be deduced that the dimer interface is located between the 30S subunits of two interacting monomers. Several 2D monomer projection maps were obtained, due to their rather globular shape. The projection map that was most similar to that of the dimer was selected (Figs 1 and 2). To facilitate comparisons between ribosome monomer and dimer, we overlaid the densities that can be attributed to the large 50S subunit of the monomer and dimer in yellow (Fig. 1D3) and red (Fig. 1D4) and the densities attributed to the smaller 30S subunits in pink and blue respectively. These surfaces were superimposed in Fig. 1D5. The orientation of the 50S subunits is largely comparable, whereas a large difference between the views of the 30S subunits can be observed. A simple explanation for this difference is a rigid body rotation of at least 30° about a fixed axis of the 30S subunit relative to the 50S subunit upon dimerization as is indicated in Fig. 1D5. At the level of the resolution of the 2D map, which is about 1.5 nm, possible further and smaller conformational changes cannot be seen. Nevertheless, the change in the relative position of the small and large subunits of the ribosome monomers in the dimer is quite dramatic.

To identify possible protein factor(s) involved in *L. lactis* ribosome dimerization, the 70S and 100S ribosome fractions were subjected to a proteomic analysis. $YfiA^{Ll}$ and 8 other proteins were co-purified in the 100S fraction only. The latter 8 are abundant proteins and include the pyruvate dehydrogenase components PdhB and PdhC, the

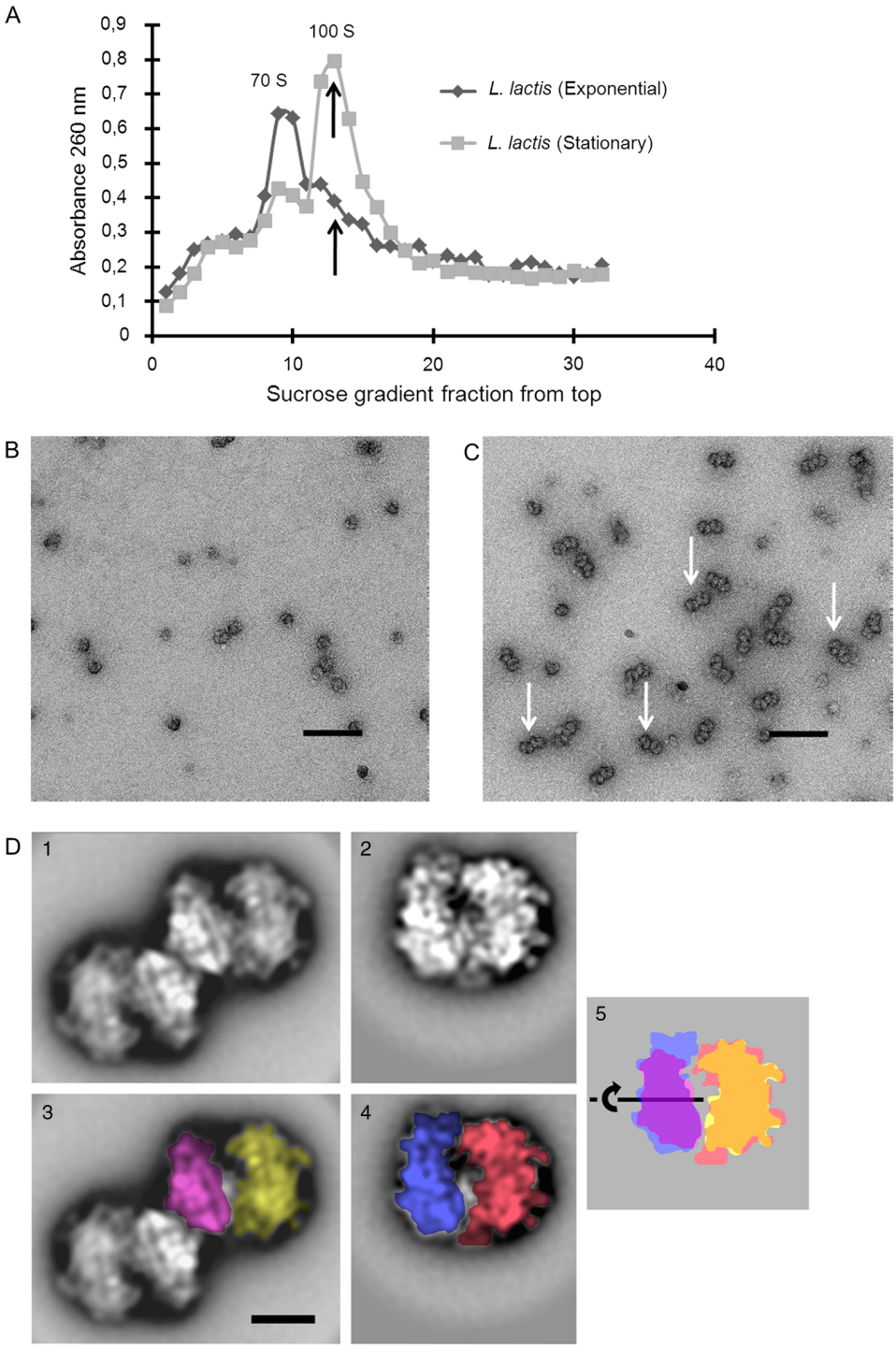

Fig. 1. *L. lactis* forms ribosome dimers (100S) in the stationary phase.
A. Profiles after sucrose density gradient centrifugation of ribosomes isolated from *L. lactis* in the exponential and stationary phases of growth. Peaks of 70S and 100S ribosome particles are indicated, as are the samples taken for electron microscopy analysis of both profiles (fraction 13, black arrows).
B and C. Electron microscopy images of the samples taken in (A) of cells harvested in the exponential or stationary phase respectively. White arrows in (C) indicate ribosome dimers.
D. Corresponding projection maps of dimeric (1) and monomeric (2) ribosomes. The visible surface areas of both projections (3 and 4) were superimposed (5), with the large 50S subunit marked in yellow/red and the small 30S subunit coloured pink/blue. The difference in projected area of the 30S subunit is best explained by a rotation shown in (5) of the 30S subunit along its interface with the 50S subunit in the monomer. The scale bars in B and C represent 100 nm, which in D denotes 10 nm, for all 5 images.

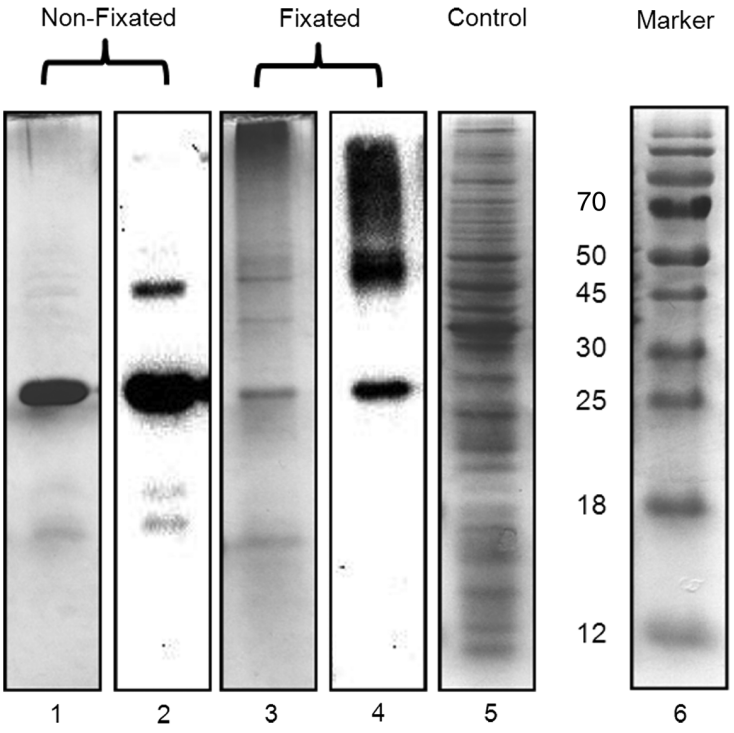

Fig. 2. Identification of interaction partners of YfiA^{LI}. *L. lactis* cells overexpressing Strep-tagged YfiA^{LI} were grown for 7 h in GM17 at 30°C, harvested and disrupted after fixation or not with 4% paraformaldehyde. The protein extracts were purified using Strep-tag affinity column chromatography and subjected to SDS-12% PAGE and Coomassie staining (panels 1, 3, 5 and the lane with the protein molecular weight marker are all from the same gel) and immune-detection using anti-Strep-tag antibodies (panels 2 and 4). The 45 kDA protein in Fig 2 is an unknown protein of *L. lactis* that reacts with the anti-Strep tag antibody (J. Kok *et al.*, unpublished). Each gel lane was sliced into 12 pieces. These were numbered according to the approximate molecular weight of the proteins expected in the slice, as estimated from the marker lane. For the protein identification results, see Table S1.

ATP-dependent nuclease subunit A (RexA), the putative cation transporter Llmg_0322, the conserved hypothetical protein PrsA, protein RplL, the HU-like DNA binding protein HllA and glutamine synthetase GlnA. Thus we investigated YfiA^{LI} further as the other 8 proteins were considered to be false positives.

YfiA from *L. lactis* (YfiA^{LI}) contains 185 amino acid residues. Based on its sequence it is predicted to consist of two domains. The N-terminal domain (residues 1–126) is distantly related to HPF^{Ec} and YfiA^{Ec}, but YfiA^{LI} is almost twice as long as the two *E. coli* proteins (Fig. S1). The C-terminal domain does not have a counterpart in *E. coli*. Conversely, *L. lactis* does not have a homologue of RMF^{Ec} nor additional HPF or YfiA components. Proteins similar in length, sequence and domain structure as YfiA^{LI} are present in other Gram-positive bacteria, including Streptococcaceae and *Staphylococcus aureus* (Ueta *et al.*, 2013; Fig. S1). Also these bacterial species do not have orthologues of *E. coli rmf* and *hpf*.

Interactions partners of L. lactis *YfiA*

In order to investigate whether or not YfiA^{LI} was indeed the only protein involved in ribosome dimerization, N-terminal Strep-tagged YfiA^{LI} (Strep-YfiA^{LI}; 22.8 kDa) was overexpressed in *L. lactis* and purified by Strep-Tactin purification. Strep-YfiA^{LI} was used to identify possible interaction partners in a pull-out assay using total cell lysates. A protein of the expected size (approximately 26 kDa) was visible on a Coomassie-stained SDS 12%-PAA gel and on a Western blot after immunolabelling with anti-Strep antibodies (Fig. 2, lanes 1 and 2). A second unidentified protein with a size of approximately 45 kDa is an unidentified protein from *L. lactis* that reacts with anti-Strep antibodies (J. Kok *et al.*, unpublished). When the cells were treated with 4% paraformaldehyde (PFA) prior to harvesting and subsequent affinity purification, a smear of proteins with sizes ranging from 50 kDa to 150 kDa was detected by Coomassie staining and immunodetection (Fig. 2, lanes 3 and 4). The lower amount of Strep-YfiA observed after PFA-fixation and the appearance of a smear of bands with apparent high molecular weight indicate that Strep-YfiA^{LI} forms complexes with other macromolecules. The PFA cross-linked and non-cross-linked affinity-purified proteins were identified by in-gel trypsin digestion followed by LC-MS/MS. As a control, a similar area of an SDS-PAA gel (lane 5 in Fig. 2), containing the non-cross-linked proteins from stationary-phase wild type *L. lactis* cells was examined. Proteins found in the smear in lane 3, which showed an apparent shift of molecular weight as compared with their position in the control lane 5 were identified as interaction partners. In the fixated sample, a number of proteins involved in protein synthesis (e.g. EF-Tu, RpsA, EF-G and IF-2) were found to be associated with Strep-YfiA^{LI} (Table S1). In addition, some enzymes from the central metabolism, like PdhC and PdhD, were identified. These components of the pyruvate dehydrogenase complex are known in *E. coli* to co-purify with rRNA modification enzymes as

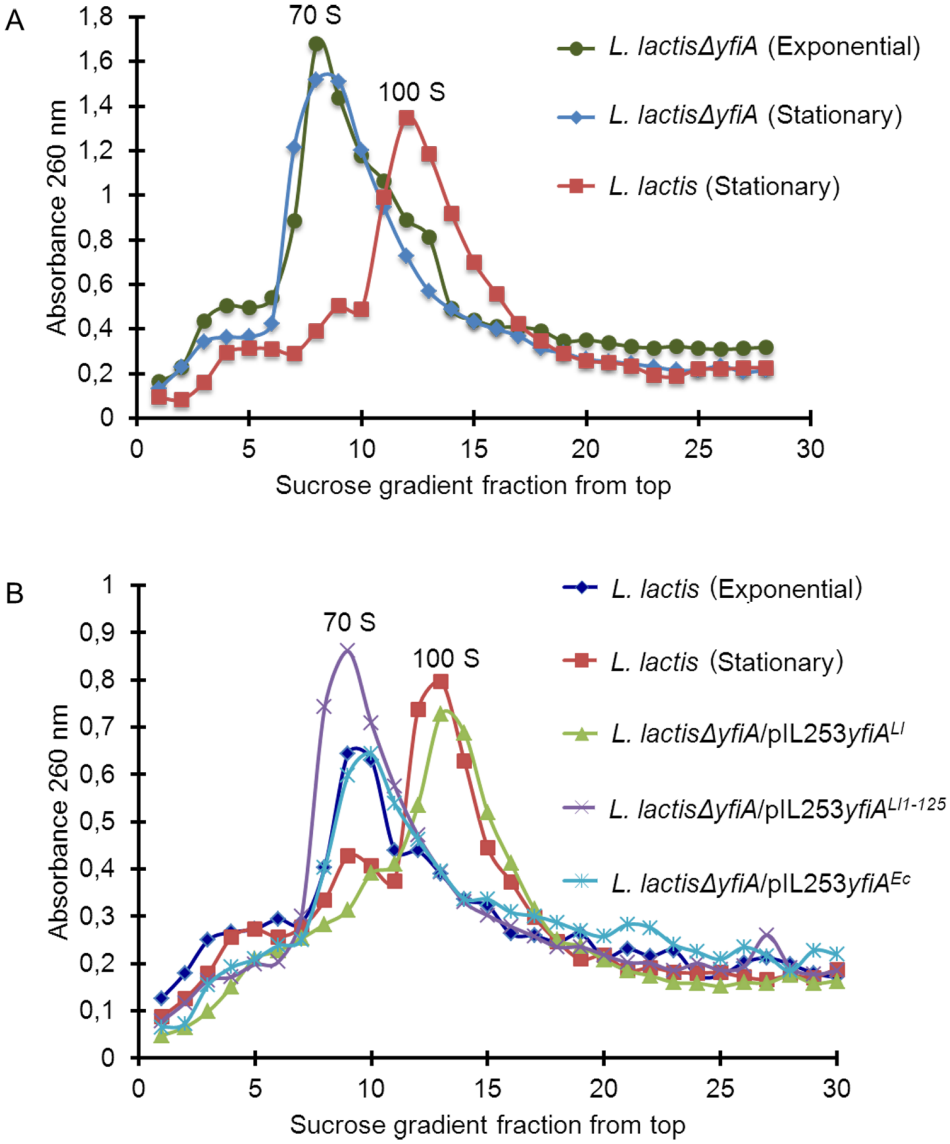

Fig. 3. YfiA^{LI} is necessary for ribosome dimerization. Sucrose density gradient centrifugation profiles of ribosomes isolated from (A) *L. lactis*Δ*yfiA* cells grown to exponential or stationary phase and *L. lactis* wild type grown to stationary phase. (B) *L. lactis* grown in exponential or stationary phase and *L. lactis*Δ*yfiA* complemented with the indicated YfiA^{LI} variants expressed from plasmid pIL253 and growing in stationary phase.

well as with the 50S ribosome subunit (Jiang *et al.*, 2006; Sergiev *et al.*, 2012). As all the proteins identified were abundant, they seem to bind non-specifically to YfiA^{LI}. None of the proteins identified by MS showed sequence identity with the *E. coli* proteins involved in dimerization, RMF^{Ec} or HPF^{Ec}, suggesting that YfiA^{LI} accomplishes the same function on its own.

L. lactis *YfiA is essential for ribosome dimerization*

In *L. lactis*Δ*yfiA* cells, 100S ribosome particles were neither observed in exponential nor in stationary phase-grown cells (Fig. 3A; 100S ribosomes from wild type stationary phase-grown cells are shown for comparison). Ribosome dimerization was restored by complementing the *yfiA* null strain *in trans* with plasmid pIL253*yfiA*^{LI}, in which *yfiA*^{LI} was expressed under the control of the constitutive lactococcal promoter P32 (Fig. 3B). We did not observe a difference in the sucrose density profiles of ribosomes isolated from wild type cells containing pIL253 (vector control) or pIL253*yfiA*^{LI}, both grown to stationary phase, indicating that *yfiA* gene copy number does not affect the ribosome dimerization. As a negative control *yfiA*^{Ec} was expressed in *L. lactis*Δ*yfiA* cells using plasmid

pIL253 or the empty plasmid, and indeed ribosome dimers were not observed (Fig. 3B and Fig. S2). Also, a C-terminally truncated variant of YfiA^{LI}, YfiA$^{LI1\text{-}125}$, did not restore the ribosome dimerization defect of *L. lactis*Δ*yfiA* (Fig. 3B). Synthesis of both full-length and truncated versions of all the proteins was verified by mass spectrometry (data not shown). Next, we analysed by mass spectrometry fraction 14 of the ribosome profiles of all 5 strains (Fig. 3B). YfiA^{LI} was detected in all cases with the exception of fraction 14 from the Δ*yfiA* strain. Full-length YfiA^{LI} and C-terminally truncated YfiA^{LI} were indeed associated with ribosomes, as shown for *L. lactis*Δ*yfiA*/pIL253*yfiA*^{LI} and *L. lactis*Δ*yfiA*/pIL253*yfiA*$^{LI1\text{-}125}$ respectively. This experiment shows that YfiA$^{LI1\text{-}125}$, lacking the C-terminal domain, can associate with ribosomes but does not elicit the conformational change required for dimer formation. Thus, the C-terminal domain of YfiA^{LI} is essential for dimerization and may assume a role similar to that of RMF^{Ec} in *E. coli*, while the N-terminal domain of YfiA^{LI} is equivalent to HPF^{Ec}. RMF^{Ec} is essential for dimerization of ribosomes in *E. coli*, which results in the formation of the 90S ribosome complex. This complex is then further stabilized by HPF^{Ec} to form the 100S dimer (Ueta *et al*., 2008). HPF^{Ec} alone cannot dimerise *E. coli* ribosome.

To determine whether or not YfiA^{LI} can take over the function of both *E. coli* proteins, *in vitro* reconstitution of ribosome dimerization was performed using high salt-washed (monomeric) ribosomes from exponentially growing *L. lactis* and *E. coli* cells. The *L. lactis* ribosomes were incubated with purified YfiA^{LI} tagged with an N-terminal Strep-tag (Strep-YfiA^{LI}) or a C-terminal His10-tag (YfiA^{LI}-His10). In both cases 70S and 100S ribosomes were observed after sucrose gradient density centrifugation (Fig. 4A) and subsequent EM (Fig. 4C and D). Strep-YfiA^{LI} was also capable of dimerizing *E. coli* ribosomes (Fig. 4B and E). Thus, YfiA^{LI} (independently from the position or type of affinity tag) can dimerise ribosomes *in vitro*. To study whether the C-terminal domain of *L. lactis* YfiA, YfiA$^{LI126\text{-}185}$ (the last 59 amino acid residues of the protein) can indeed function in a way similar to RMF in *E. coli*, C-terminally His10-tagged versions of YfiA$^{LI126\text{-}185}$, HPF^{Ec} and RMF^{Ec} were overexpressed in *E. coli* and purified by metal-affinity chromatography. *L. lactis* ribosomes purified from exponential phase cells formed dimers *in vitro* when they were incubated with HPF^{Ec}-His plus RMF^{Ec}-His or YfiA$^{LI126\text{-}185}$-His plus HPF^{Ec}-His, demonstrating that indeed the C-terminal domain of YfiA^{LI} is a functional paralogue of RMF^{Ec} (Fig. 5).

Functional role of L. lactis *YfiA*

To determine the physiological role of YfiA^{LI}, the growth in rich and chemically defined medium of wild type *L. lactis*, its isogenic *yfiA* mutant and the complemented strain *L. lactis*Δ*yfiA*/pIL253*yfiA*^{LI} were evaluated. No differences were observed in the growth rate of the strains (data not shown). Their growth after starving the cells for glucose for several days was also tested. Up to 5 days without glucose no significant differences were seen between the three strains, but the growth of *L. lactis*Δ*yfiA* was reduced relative to wild type upon longer starvation (Fig. 6, compare subpanels A1, A2 and A3). While the wild type *L. lactis* cells were able to resuscitate and grow again even after 18 days of starvation (subpanel A3), the viability of the *yfiA* mutant dropped to zero within 14 days. The complemented strain survived for 16 days (subpanel A2) but was not able to resuscitate after 18 days of growth (subpanel A3). Moreover, after prolonged starvation, the lag times before re-growth of *L. lactis*Δ*yfiA* and *L. lactis*Δ*yfiA*/pIL253*yfiA*^{LI} increased more than that of wild type *L. lactis* (Fig. 6B).

Discussion

Oligomerization of ribosomes was first observed more than 50 years ago and interpreted as the aggregation of 70S ribosomes (Tissieres and Watson, 1958). We now know that this 'aggregation' is a specific, physiologically relevant process. The molecular and structural basis for the formation of 100S particles has recently been addressed (Kato *et al*., 2010; Polikanov *et al*., 2012). The picture that emerges is that one (*L. lactis*, this work) or more (in case of *E. coli*) small proteins bind to and cause conformational changes in the 30S ribosomal subunit, which increases the affinity of self-association of this subunit. The current study proves that ribosome dimerization takes place when *L. lactis* cells enter the stationary phase. Our *in vivo* and *in vitro* work revealed that *L. lactis* YfiA is necessary and sufficient for ribosome dimerization in *L. lactis*. Heterologously overexpressed *E. coli* YfiA^{Ec} did not rescue the *yfiA* null mutation in *L. lactis*. Also, C-terminally truncated YfiA$^{LI1\text{-}126}$ does not allow ribosome dimer formation, indicating that important dimerization functions in YfiA^{LI} are located in the C-terminus of the protein. In fact we show that the C-domain of YfiA^{LI}, YfiA$^{LI126\text{-}185}$, in combination with *E. coli* HPF can dimerise *L. lactis* ribosomes *in vitro*. *L. lactis* YfiA is an example of the so-called long HPF type (Ueta *et al*., 2013). It resembles the HPF protein from *S. aureus*, which has been shown to be involved in ribosome dimerization (Ueta *et al*., 2010). Apart from the N-terminal domain that is conserved in all HPF homologues (Fig. S1), both proteins contain an extended, long and mutually similar C-terminal domain (Ueta *et al*., 2008). By contrast, YfiA^{Ec} contains only a short C-terminal extension that cannot elicit ribosome dimerization but, instead, prevents binding of RMF^{Ec} to its actual site in the ribosome. Thus, YfiA^{Ec} precludes ribosome dimerization by interfering with RMF^{Ec} dependent 90S formation (Polikanov *et al*., 2012). In the present study, we show that the extended

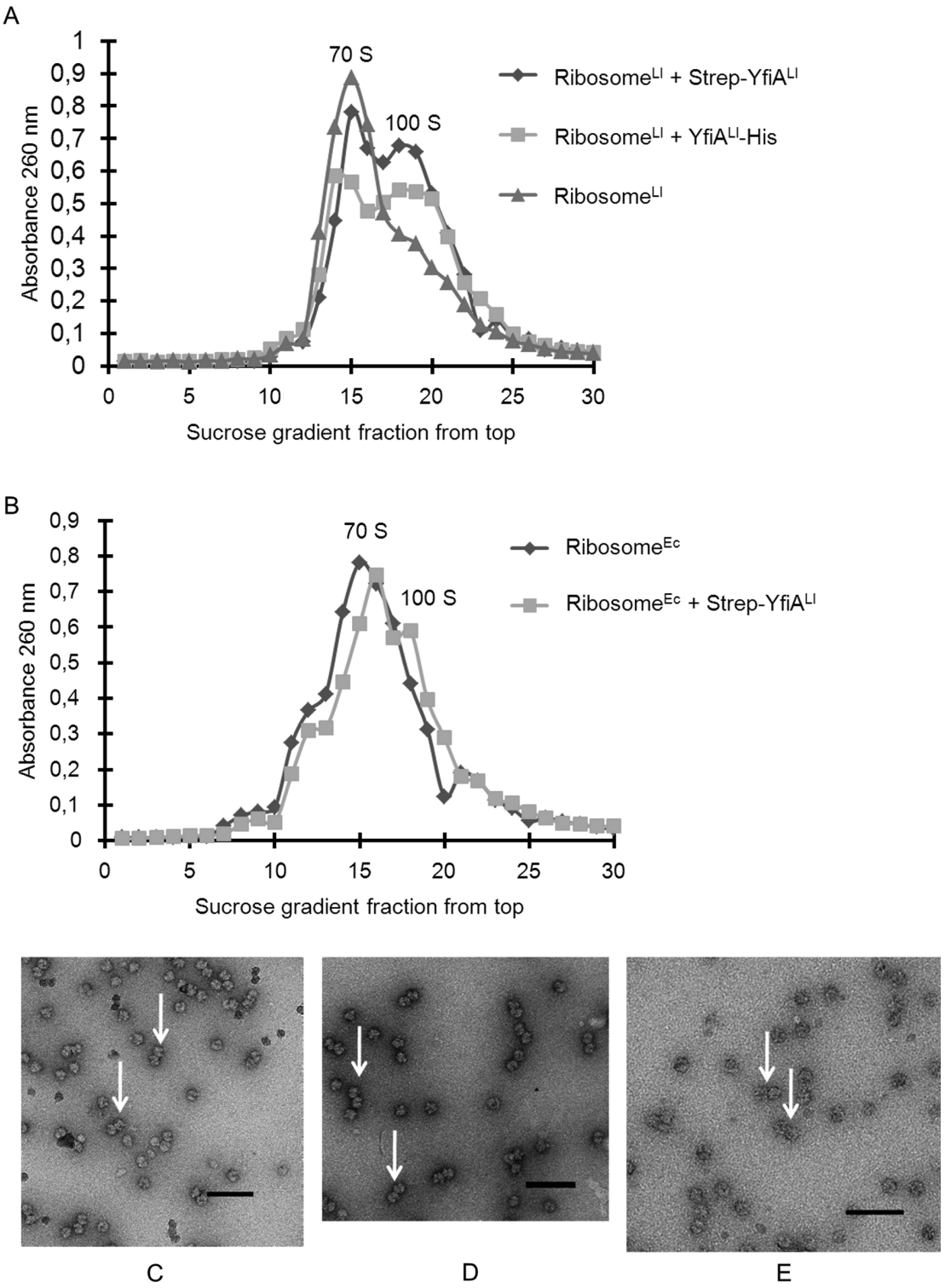

Fig. 4. Sucrose density gradient centrifugation profiles of ribosome dimerization *in vitro*. (A) *L. lactis* ribosomes (Ribosome^{LI}) incubated with Strep-YfiA^{LI} or YfiA^{LI}-His and (B) *E. coli* ribosomes (Ribosomes^{Ec}) incubated with Strep-YfiA^{LI}. Negative-stain electron micrographs of *L. lactis* ribosomes incubated with (C), Strep-YfiA^{LI}; (D), YfiA^{LI}-His10 and (E), *E. coli* ribosomes incubated with Strep-YfiA^{LI}. The scale bars on the electron micrographs represent 100 nm.

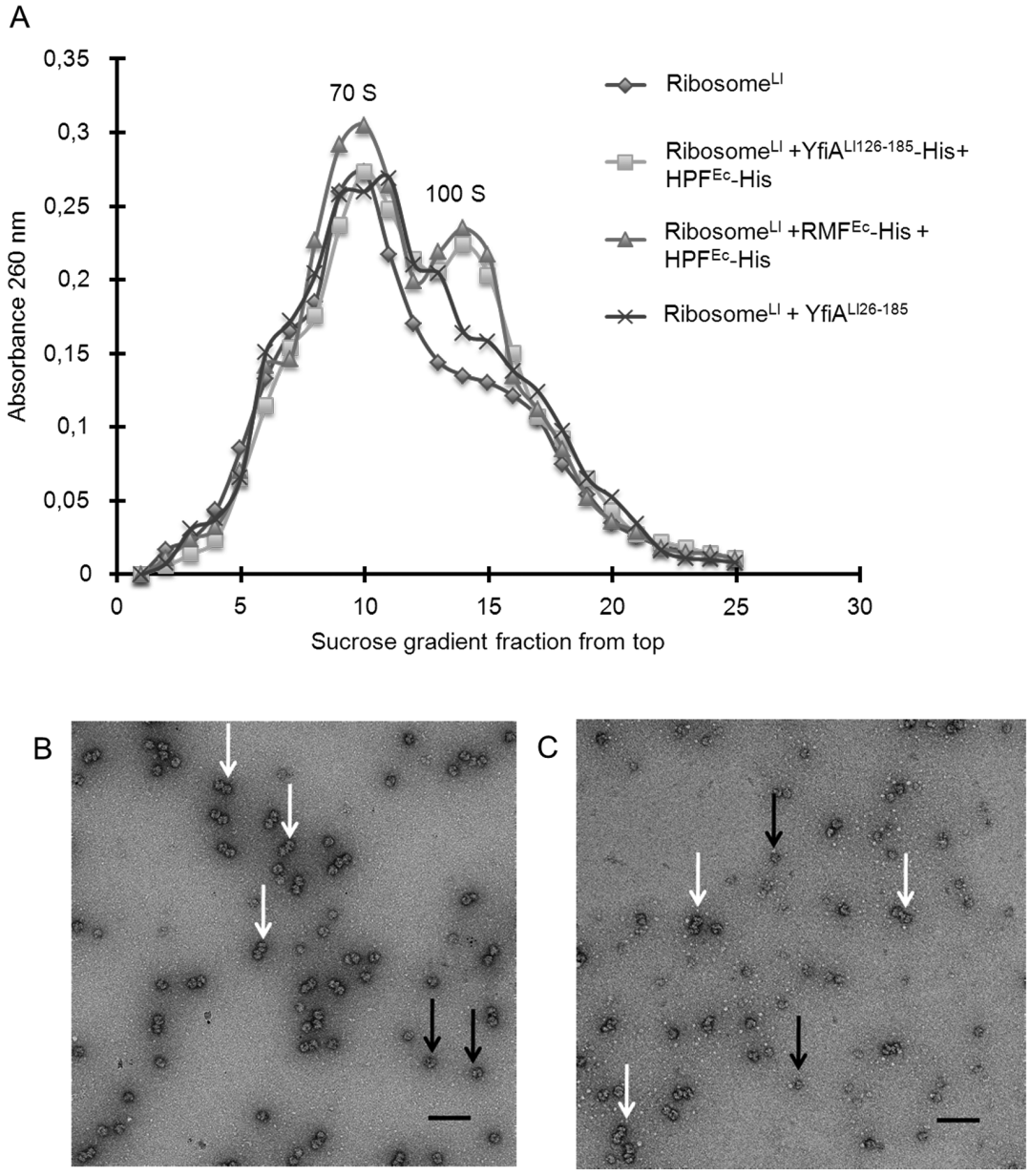

Fig. 5. YfiA^{LI} is sufficient for ribosome dimerization. (A) Sucrose density gradient centrifugation profiles of *L. lactis* ribosomes incubated *in vitro* with YfiA$^{LI126-185}$-His alone, with YfiA$^{LI126-185}$-His plus HPF^{Ec}-His, and with RMF^{Ec}-His plus HPF^{Ec}-His. Negative-stain electron micrographs of *L. lactis* ribosomes incubated *in vitro* with (B) YfiA$^{LI126-185}$-His plus HPF^{Ec}-His or with (C) RMF^{Ec}-His plus HPF^{Ec}-His. White and black arrows indicate ribosome dimers and monomers respectively. The scale bars on the micrographs represent 100 nm.

C-terminal domain in YfiA^{LI} performs a completely opposite function as it triggers ribosome dimerization. Importantly, YfiA^{LI} can dimerise *L. lactis* ribosomes without requiring other protein factors and can substitute for HPF^{Ec} plus RMF^{Ec} in the *in vitro* dimerization of *E. coli*. The heterologous reconstitution of *E. coli* ribosome dimers with *L. lactis* YfiA indicates that the C-terminus of YfiA^{LI} functionally resembles *E. coli* RMF even though their sequences are unrelated. Thus, indeed YfiA^{LI} belongs to the long HPF type (Ueta *et al.*, 2013) and we re-annotate it as LIHPF (for *Lactococcus lactis* hibernation promotion factor).

Our EM analysis shows that a large conformational change takes place in the position of the 30S subunit relative to the 50S subunit upon ribosome dimerization in *L. lactis* (Fig. 1C). In *E. coli* it has been shown that RMF^{Ec} is involved in repositioning of the 30S subunit relative to the 50S subunit in the *E. coli* 100S dimer. The *E. coli* ribosomal proteins S2, S3 and S5 are involved in

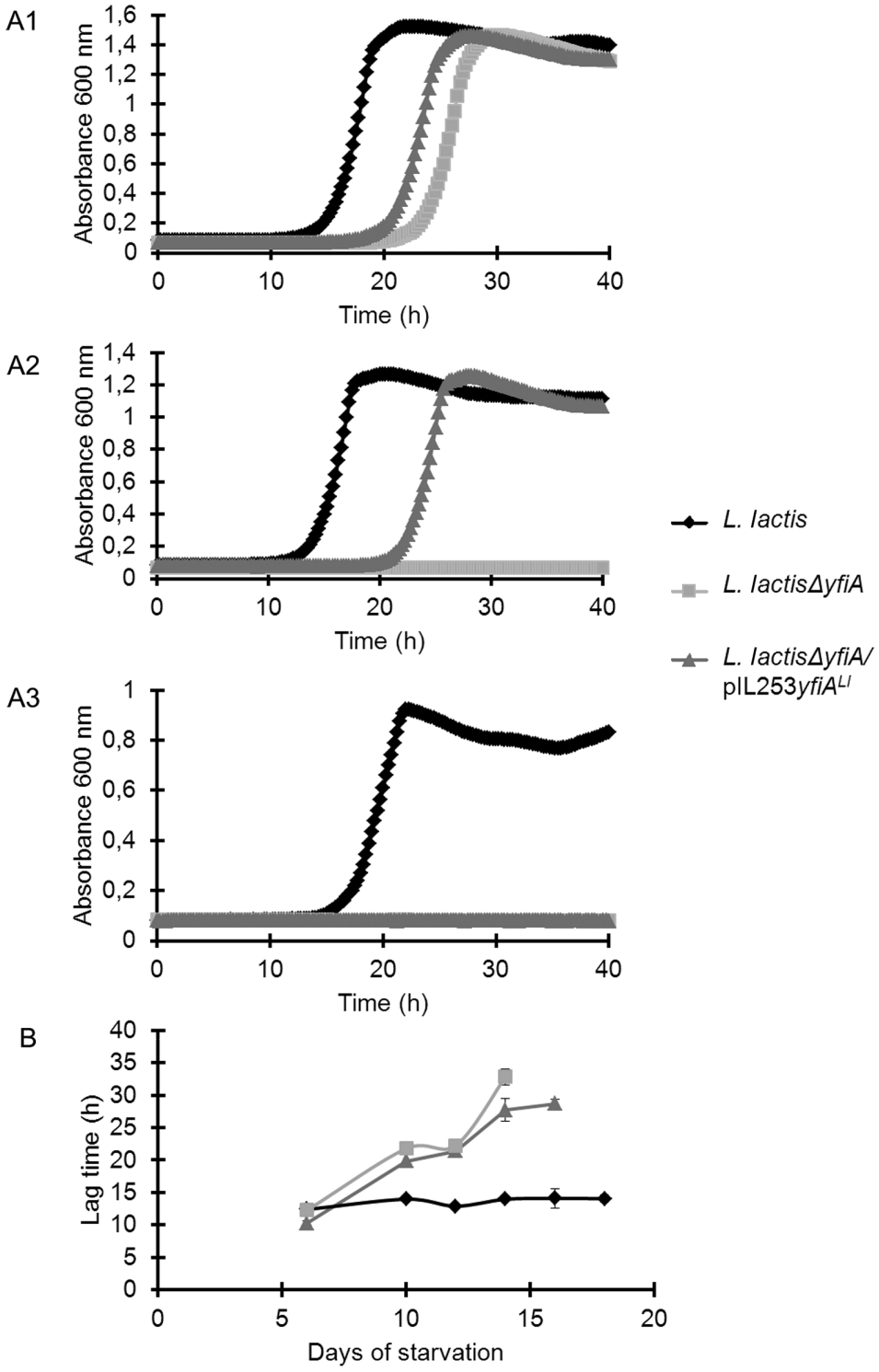

Fig. 6. A. Resuscitation patterns of *L. lactis*, *L. lactisΔyfiA* and *L. lactisΔyfiA*/pIL253*yfiA*^{LI}. All three isogenic strains were grown and subsequently starved for prolonged periods of time in a chemically defined medium (CDM) at 30°C. Then, the strains were resuspended and diluted 100-fold in fresh CDM-glucose medium (with erythromycin for the plasmid-containing strain) in microtitre plates, after which the development of OD_{600} of the cultures was followed for 48 h. Subpanels A1, A2 and A3 indicate growth after 10, 16 and 18 days of starvation. B. Lag time of re-growth in fresh CDM-glucose medium as a function of days of starvation of *L. lactis*, *L. lactisΔyfiA* and *L. lactisΔyfiA*/pIL253*yfiA*^{LI}. Data plotted in (B) are from the cultures shown in (A) and data not shown. Data shown are obtained from 4 independent cultures.

formation of the dimerization domain (Kato *et al*., 2010). Interestingly, a conformational change between the 30S and 50S subunits to the extent seen in *L. lactis* is not observed in *E. coli* (Kato *et al*., 2010). Interpretation of negatively stained projection maps in greater detail is risky, as the method of negative staining is prone to artifacts like dehydration and flattening of the protein. Therefore, to allow for a more detailed comparison between the two species, 3D cryo-EM reconstructions with a much higher resolution are currently being performed.

Various factors known to interact with the ribosome and involved in translation and ribosome recycling were co-purified with Strep-YfiA^{Ll}. We identified initiation factor 2 (InfB), a protein that allows fMet-tRNA and 30S and 50S subunits to form a 70S ribosome (Myasnikov *et al*., 2009), and elongation factor Ts, a protein catalysing the release of guanosine diphosphate from EF-Tu. Also, protein-folding factors such as DnaK, GroEL and trigger factor TG co-purified with Strep-YfiA^{Ll}. As these proteins and the co-purified glycolytic enzymes are all very abundant in the cytosol (Steen *et al*., 2010) they might reflect a-specific binding. Possible exceptions are the proteins of the pyruvate dehydrogenase complex as both in *B. subtilis and S. aureus* ribosomes are protected from degradation by trypsin when they are bound to the cytoplasmic membrane in a complex with pyruvate dehydrogenase (Adler and Arvidson, 1987). It is an intriguing possibility that the membrane-bound complex serves as a 'parking lot' for hibernating ribosome dimers.

The physiological role of ribosome dimerization has remained enigmatic. In *E. coli*, inactivation of YfiA^{Ec} stimulates ribosome dimerization as the protein interferes with the binding of RMF^{Ec} and HPF^{Ec} to the ribosomes. The *E. coli yfiA*::*Km* mutant appears to be somewhat more viable in the stationary phase. The enhanced viability of this strain has been explained by increased protection of 100S (as compared with 70S) ribosomes against degradation by RNA hydrolases (Zundel *et al*., 2009; Ortiz *et al*., 2010). As anticipated, *L. lactis*Δ*yfiA* does not have a phenotype in rapidly growing cells, but the viability of the mutant is decreased when it is starved for carbon and energy sources. The complemented *L. lactis*Δ*yfiA* strain survives the starvation regime in a manner similar to the wild type, except that its lag phase is increased. We attribute the prolonged lag phase to the antibiotic required for plasmid maintenance in the mutant. In the presence of erythromycin, cells deprived of metabolic energy have a longer lag time when transferred to fresh medium than cells cultivated in the absence of the antibiotic.

We propose that ribosome dimerization prevents the synthesis of incomplete proteins under conditions that the availability of carbon and energy source (and thus aminoacyl-tRNAs) is limiting, and thereby saving metabolic energy. Such a mechanism would be highly beneficial for *L. lactis*, as the organism does not adjust its ribosome content to the need of protein synthesis, that is, at low growth rates the cell has an excess capacity of ribosomes (A. Goel *et al*., manuscript in preparation). By reversibly inactivating ribosomes, the cells do not waste resources on unnecessary protein synthesis and they are prepared for conditions of fast growth as soon as sufficient nutrients become available again.

In conclusion, we show that *L. lactis* has a dual-domain protein, YfiA^{Ll}, which we re-annotate as LlHPF that elicits a major conformational change in ribosomes and triggers their dimerization. The C-terminal domain of YfiA^{Ll} has a role similar to that of RMF from *E. coli*, even though the two proteins are not homologous. The YfiA^{Ll} C-terminal domain elicits a conformation change in the 30S subunits, which allows two 70S ribosomes to interact with each other and form the 100S dimer. The functional role of YfiA^{Ll} is apparent under energy-starving conditions. The phenomenon of ribosome dimerization is here proposed to be a mechanism to adjust protein synthesis capacity by allosterically controlling the activity of ribosomes.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table S2. *E. coli* was grown aerobically at 37°C in TY medium (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl). *L. lactis* strains were grown as standing cultures at 30°C in M17 medium (Difco Laboratories, Detroit, MA, USA) supplemented with 0.5% (w/v) glucose (GM17). Solid media contained 1.5% agar. Chloramphenicol (5 μg ml^{-1}) and erythromycin (120 μg ml^{-1} for *E. coli* and 2.5 μg ml^{-1} for *L. lactis*) were added when required. For determination of the functional role of YfiA^{Ll}, *L. lactis* strains were grown as standing cultures (two biological replicates for each) in chemically defined medium (CDM) (Poolman and Konings, 1988) supplemented with 0.5% (w/v) glucose and 2.5 μg ml^{-1} erythromycin at 30°C. Cells were taken from this standing culture, after every 2 days until day 18 and re-inoculated with fresh CDM-glucose and growth was recorded in 96 well microtitre plate at 30°C for 24 h (Biotec Powerwave 340).

General DNA techniques

Plasmid DNA was isolated using a High Pure Plasmid Isolation Kit and protocol (Roche Applied Science, Indianapolis, IN, USA). Chromosomal DNA was isolated from *L. lactis* as described previously (Johansen and Kibenich, 1992). Polymerase chain reactions (PCR) were either performed with the Phusion enzyme (Finnzymes, Espoo, Finland) or with a modified version of it, named PfuX7 (Nørholm, 2009). PfuX7 yields a uracil excision-ready PCR fragment that was subsequently ligated with a mixture of uracil DNA glycosidase and DNA glycosylase-lyase endo VIII, commercially available as USER, using the manufacturer's instructions (New England Biolabs,

Ipswich, MA, USA). Colony PCR was performed with Taq Polymerase (ThermoFisher, Waltham, MA, USA). Primers listed in Table S3 were purchased from Biolegio BV (Nijmegen, the Netherlands). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Applied Science) according to the protocol of the supplier. DNA electrophoresis was performed in 1× TBE buffer (90 mM Tris-HCl pH 8.3, 90 mM boric acid, 2 mM EDTA) in 1% agarose gels with 0.5 μg ml^{-1} ethidium bromide. Electrotransformation of *L. lactis* was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA, USA). Nucleotide Sequencing reactions were done at MacroGen (Seoul, Korea).

L. lactis *YfiA overexpression and protein purification*

The *yfiA* gene was amplified by PCR using *L. lactis* MG1363 chromosomal DNA as the template and primers Pr8 and Pr9 (Table S3). The PCR product, consisting of the *yfiA* gene extended at the 5′-end with the codons for the Strep-tag (Skerra and Schmidt, 2000), was purified, digested with NcoI and XbaI and ligated into pNZ8048 (De Ruyter *et al.*, 1996) cut with the same enzymes. The same strategy was used to clone the *yfiA* gene without the Strep-tag sequence (primers Pr7 and Pr9). The resulting plasmids (pNZ*strepyfiA* and pNZ*yfiA* respectively), in which *strepyfiA* and the *yfiA* were under the control of the nisin-inducible promoter P_{nisA}, were obtained in *E. coli* and subsequently introduced in *L. lactis* NZ9000 (Kuipers *et al.*, 1998). Nucleotide sequences were confirmed by nucleotide sequence analysis. YfiA^{LI} and Strep-tagged YfiA^{LI} (Strep-YfiA^{LI}) were overexpressed using the nisin-inducible system (NICE) (De Ruyter *et al.*, 1996). Filter-sterilized culture supernatant of the nisin-secreting strain *L. lactis* NZ9700 was used as a source of nisin. Overnight cultures of *L. lactis* NZ9000 carrying either pNZ*strepyfiA* or pNZ*yfiA* were diluted 100-fold in 1 l of fresh GM17 medium with 5 μg ml^{-1} chloramphenicol and incubated at 30°C. Nisin-containing supernatant was added (1:500 v/v) when the OD_{600} of the culture had reached 0.8. After 4 h of further incubation, the cells were pelleted (7000 *g* for 10 min), resuspended in either 10 ml phosphate-buffered saline (PBS) or in 10 ml PBS with 0.6% paraformaldehyde and incubated at 37°C for 20 min. Cells were centrifuged (7000 *g* for 10 min at 4°C), washed once with buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.0), resuspended in 10 ml wash buffer and stored at –80°C. Cells were thawed at room temperature, and treated with 10 mg ml^{-1} lysozyme and Complete Mini Protease Inhibitor (Roche Applied Science) for 60 min at 30°C. Subsequently, 0.1 g DNase I and 10 mM $MgSO_4$ were added to cell suspension, and the lysozyme-treated cells were broken using a probe sonicator; 5 cycles of sonication for 45 s with 15 s of cooling on ice in between the cycles (Aminco, Silver Springs, MD, USA). The suspension was centrifuged at 9000 *g* (15 min, 4°C). Strep-YfiA^{LI} was purified to homogeneity using a Strep-tactin Sepharose column according to the manufacturer's instructions (IBA-GmbH, Göttingen, Germany). Samples from each step in the purification protocol were analysed by sodium dodecylsulphate 12%-polyacrylamide gel electrophoresis (SDS 12%-PAGE) (Laemmli, 1970) and Western hybridization using anti-Strep-tag antibodies (IBA-GmbH). The concentration of purified protein was determined via spectroscopy (Nanodrop, ThermoFisher). Protein (100 μM) was kept at –80°C in 10% glycerol, 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin.

Overexpression and purification of E. coli *HPF and RMF and the* L. lactis *C-terminal domain of* L. lactis *YfiA*

The *hpf* and *rmf* genes were amplified using primers Pr17/Pr18 and Pr19/Pr20 respectively (Table S3). The gene fragment corresponding to the C-terminal domain of YfiA^{LI} (YfiA$^{LI126\text{-}185}$, encompassing the 59 C-terminal amino acids of the protein) was amplified using primers Pr21/Pr22. The three amplified fragments were cloned using ligation-independent cloning into a pBADcLIC overexpression vectors, extending the cloned genes with a tag that adds 10 histidine residues to the C-terminus of the encoded proteins (Geertsma and Poolman, 2007). The cells carrying an overexpression construct were grown in LB media at 37°C and induced with 0.01% arabinose for 3 h once the cells had reached an OD_{600} of 0.6. The cells were harvested, washed and resuspended in lysis buffer (20 mM Tris-HCl pH 7.6, 100 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol). Cells were lysed, using the Constant cell disrupter system (Constant Systems, UK) at 9000 psi. Subsequently, the lysate was centrifuged at 25 000 *g* for 30 min to remove cell debris and then at 225 000 *g* for 1 h to remove membranes. The supernatant was filtered through a 0.2 μm membrane filter and loaded onto TALON resin (Clontech, CA). The resin was washed with high-salt buffer (20 mM Tris-HCl, pH 7.6, 1 M NH_4Cl, 10 mM $MgCl_2$, 1 mM 2-mercapthoethanol) to remove non-specifically bound proteins. The his-tagged proteins were eluted from the column with a linear gradient of 0–300 mM imidazole. Fractions containing the desired protein were combined and concentrated and used for the *in vitro* assays.

Construction of a L. lactis yfiA *deletion and complementation strains*

Upstream and downstream regions of *yfiA* of *L. lactis* MG1363 were, PCR-amplified using primer pairs Pr3/Pr4 and Pr5/Pr6 (Table S2), respectively, were inserted in the integration vector pCS1966 (Solem *et al.*, 2008), amplified with Pr1 and Pr2, and ligated with USER as described above. The resulting plasmid, pCS1966Δ*yfiA*, was obtained in *E. coli* and introduced into *L. lactis* MG1363 to allow integration via single crossover homologous recombination. A *L. lactis* integrant carrying the pCS1966 construct was selected on GM17 plates with chloramphenicol. Screening for subsequent plasmid excision was done on plates containing 5-fluoroorotic acid by selecting against the *oroP* gene on pCS1966 (Solem *et al.*, 2008). A mutant carrying a clean knockout of *yfiA* was obtained and confirmed by PCR and nucleotide sequence analysis. Complementation of *L. lactis*Δ*yfiA* was done with three different plasmids: pIL253*yfiA*^{LI}, pIL253*yfiA*$^{LI1\text{-}125}$ and pIL253*yfiA*^{Ec}. The *yfiA* genes from *L. lactis* and *E. coli* were amplified using primer pairs Pr10/Pr11 and Pr12/Pr13 respectively. A truncated version of *L. lactis* YfiA lacking the 59 C-terminal amino acids (YfiA$^{LI1\text{-}125}$) was amplified using primer pair Pr10/Pr14. The amplified products were ligated in the pIl253 vector (Simon and Chopin, 1988) amplified with primer pair Pr15/Pr16, employing USER enzyme. Constructs were introduced in *L. lactis*Δ*yfiA* via electrotransformation (Holo and Nes, 1989).

Isolation and purification of ribosomes

L. lactis cells were harvested by centrifugation at 7000 *g* for 10 min at 4°C after 3 h (OD_{600} = 0.5) or 7 h (OD_{600} = 2.5) of growth in GM17 at 30°C. The cell pellet was resuspended in buffer I (20 mM Tris-HCl (pH 7.6), 15 mM magnesium acetate, 100 mM ammonium acetate, and 6 mM 2-mercaptoethanol) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Subsequently, the cells were lysed by vortexing with 0.2 mg glass beads in an ice-cold Tissue lyser (Qiagen, Venlo, the Netherlands). The homogenate was centrifuged at 9000 *g* for 15 min at 4°C. The supernatant was saved on ice and the pellet was resuspended in buffer I supplemented with 1 mM PMSF. The suspension was centrifuged again under the same conditions. The combined supernatants (cell extracts) were layered onto a 30% sucrose cushion in buffer I and centrifuged in a MLA 80 rotor (Beckman, Fullerton, CA, USA) at 206 000 *g* for 3 h at 4°C. By resuspending the pellet in buffer I a crude preparation of ribosomes was obtained. To isolate ribosome monomers and dimers, this crude ribosome preparation (400 µl) was layered onto a linear 10–40% 12 ml sucrose density gradient column in buffer I and centrifuged in a SW 32.1 Ti rotor (Beckman) at 125 000 *g* for 80 min at 4°C (Maki *et al.*, 2000). Fractions of 400 µl were taken from the top of the gradient after which the absorbance at 260 nm was measured with a UV-1700 spectrometer CARY bio UV-Visible Spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). The first 1.5 ml from the top of the gradient, containing mostly small RNAs and nucleotides, was discarded and not used for the sucrose density profiles. The fractions containing the ribosome monomers or dimers were dialysed at 4°C against buffer I and prepared for mass spectrometry and EM.

In vitro *dimerization assay*

Ribosomes (10 µM) purified from exponentially growing *E. coli* or *L. lactis* were combined with 20-fold excess of purified RMF^{Ec}-His, HPF^{Ec}-His, Strep-$YfiA^{Ll}$, $YfiA^{Ll}$-His, $YfiA^{Ll126\text{-}185}$-His (elution buffer exchanged with buffer I) or combinations of these proteins. The mixtures were incubated at 37°C for 30 min after which they were layered onto a 30% sucrose cushion prepared in buffer I and centrifuged at 225 000 *g* for 3 h. The obtained pellet was resuspended in buffer I and layered onto a linear 10–40% sucrose gradient prepared in buffer 1 and centrifuged in a SW 32.1 Ti rotor at 125 000 *g* for 80 min at 4°C. The gradient was fractionated as described above.

Mass spectrometry and protein identification

For in-solution tryptic digestion, dialysed fractions containing ribosome monomers or dimers (400 µl) were precipitated overnight at –20°C, in 80% acetone. The precipitated proteins were first resuspended in 2 µl of 6 M urea and subsequently diluted to 20 µl with 100 mM triethylammonium bicarbonate (TEAB). For reduction and alkylation of the cysteine residues, the samples were incubated for 60 min at 55°C in the presence of 5 mM Tris (2-carboxymethyl) phosphine hydrochloride (TCEP) followed by the addition of 10 mM methyl methanethiosulphonate (MMTS) and incubation at room temperature for 10 min. The protein mixture was incubated overnight with 0.25 µg of trypsin (Trypsin Gold, mass spectrometry grade, Promega 10 ng μl^{-1} in 25 mM NH_4HCO_3) at 37°C. The peptide mixture was diluted with 5% formic acid and aliquots were analysed by LC-MSMS on a LTQ Orbitrap XL (Thermo Scientifics, Bremen, Germany) as described below. In-gel tryptic digestions were performed as described in Drop *et al.* (2011). Tryptic peptides were concentrated onto a pre-column (EASY-Column C18, 100 µm × 2 cm, 5 µm particle size, Thermo Scientific) and separated on a capillary column (EASY-Column C18, 75 µm × 10 cm, 3 µm particle size, Thermo Scientific) mounted on a Proxeon Easy-nLCII system (Thermo Scientific). Solutions of 0.1% formic acid in water and a 0.1% formic acid in 100% acetonitrile were used as a mobile phases. A gradient from 4% to 35% acetonitrile was performed in 75 min; at a flow rate of 300 nl min^{-1}. MS scans were acquired in the Orbitrap, in the range from 300 to 1800 m/z, with a resolution of 60 000. The 7 most intense ions per scan were submitted to MS/MS fragmentation (35% normalized collision energy) and detected in the linear ion trap.

The MS raw data were submitted to Mascot (version 2.1, Matrix Science, London, UK) using the Proteome Discoverer 1.3 analysis platform (Thermo Scientific) and searched against the *L. lactis* MG1363 proteome. Peptide tolerance was set to 20 ppm and 2.0 Da for intact peptides and fragment ions respectively; using semi-trypsin as protease specificity and allowing for up to 2 missed cleavages. Oxidation of methionine residues, deamidation of asparagine and glutamine, and cysteine modifications were specified as variable modifications according to the digestion procedure used. The MS/MS based peptide and protein identifications were further validated with the program Scaffold (version Scaffold_3.0, Proteome Software Inc., Portland, OR). Protein identifications based on at least 2 unique peptides identified by MS/MS, each with a confidence of identification probability higher than 95%, were accepted.

Transmission EM and single particle analysis

Purified ribosome monomer and dimer samples were prepared for negative staining with 2% uranyl acetate on glow-discharged carbon-coated copper grids. EM was performed on a Philips CM120 electron microscope (FEI, Eindhoven, the Netherlands) equipped with a LaB_6 cathode, operated at 120 kV. Images were recorded at 300 nm defocus with a 4000 SP 4K slow-scan CCD camera (Gatan, Pleasanton, CA, USA) at 80 000-fold magnification with a pixel size of 0.375 nm at the specimen level. GRACE software (Oostergetel *et al.*, 1998) was used for semi-automated data acquisition. A total of 23 000 wild type dimers of the stationary growth phase and 60 000 wild type monomers of the exponential growth phase were picked and analysed with the Groningen Image Processing software using standard procedures (Boekema *et al.*, 1999). Images were pretreated using a low frequency cut-off filter based on the maximum size of the particle and a high frequency cut-off filter based on the maximum resolution available in negative stain (10 Å). Images were optimized by application of conditional summing with the correlation coefficient of the final alignment step as a quality parameter to select the most homogeneous images in each class. This resulted in a dimer map consisting of 5600 particles (Fig. 1D1) and a corresponding monomer map of 4400 particles summed

(Fig. 1–2). Superimposing of the corresponding monomer and dimer was done based on visible surface area.

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Supporting information

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