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Ribosomal dimerization factor YfiA is the major protein synthesized after abrupt glucose depletion in *Lactococcus lactis*

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We analysed the response of the model bacterium *Lactococcus lactis* to abrupt depletion of glucose after several generations of exponential growth. Glucose depletion resulted in a drastic drop in the energy charge accompanied by an extremely low GTP level and an almost total arrest of protein synthesis. Strikingly, the cell prioritized the continued synthesis of a few proteins, of which the ribosomal dimerization factor YfiA was the most highly expressed. Transcriptome analysis showed no immediate decrease in total mRNA levels despite the lowered nucleotide pools and only marginally increased levels of the *yfiA* transcript. Severe up-regulation of genes in the FruR, CcpA, ArgR and AhrC regulons were consistent with a downshift in carbon and energy source. Based upon the results, we suggest that transcription proceeded long enough to record the transcriptome changes from activation of the FruR, CcpA, ArgR and AhrC regulons, while protein synthesis stopped due to an extremely low GTP concentration emerging a few minutes after glucose depletion. The *yfiA* deletion mutant exhibited a longer lag phase upon replenishment of glucose and a faster death rate after prolonged starvation supporting that YfiA-mediated ribosomal dimerization is important for keeping long-term starved cells viable and competent for growth initiation.

INTRODUCTION

Lactic acid bacteria (LAB) comprise a group of Gram-positive bacteria that is used extensively in the food industry. During adaptation to a nutrient-rich environment, LAB have developed a solely fermentative lifestyle. Apparently, the disadvantage of a low energy yield on sugar, amounting to two moles of ATP per mole glucose, is less important than the resulting rapid acidification of the surroundings, which gives the acid-resistant LAB a competitive advantage compared to other bacteria. Commercially, this acidification is exploited for production of numerous foods, e.g. fermented milk, vegetables and meat. During such a fermentation process, the bacteria will initially experience very high availability of sugars, but after the sugar is utilized, they have been proven to cope with prolonged periods of starvation, as they are able to resume growth if nutrients become available.

Lactococcus lactis is the main constituent of dairy starter cultures used worldwide for production of fermented milk products via the conversion of lactose to lactic acid. By cutting off the medium supply to a chemostate culture of *L. lactis*, Poolman and co-workers detected rapid changes in the concentration of glycolytic intermediates within the first 10 min after glucose depletion followed by disappearance of the membrane potential and the proton motive force within 90 min (Poolman *et al.*, 1987). Transcriptomic changes were later analysed following glucose depletion in a batch fermentation (Redon *et al.*, 2005b). The authors concluded that the mRNA levels were stabilized against degradation as a response to glucose starvation (Redon *et al.*, 2005a), while the overall transcription rate lowered considerably. The stabilization of mRNA was later found to coincide with a lowered expression of RNase genes (Redon *et al.*, 2005b). Purine nucleotide biosynthetic genes, belonging to the 5-phosphoribosyl-1-pyrophosphate (PRPP)-responsive PurR regulon (Jendresen *et al.*, 2012) and genes encoding ribosomal proteins, subjected to GTP-responsive stringent

regulation (Dressaire *et al.*, 2011; Rallu *et al.*, 2000) were also down-regulated (Redon *et al.*, 2005b). It thus appeared like glucose depletion, which resulted in lowered GTP availability and reduced synthesis of ribosomes.

Ribosomal hibernation is a common mechanism for bacteria to store idling ribosomes in the form of ribosomal dimers (Kline *et al.*, 2015; Puri *et al.*, 2014; Ueta *et al.*, 2010, 2013). The ability of bacterial 70S ribosomes to form 100S ribosomal dimers was first observed in the model organism *Escherichia coli* (Wada, 1998; Wada *et al.*, 1990; Yoshida & Wada, 2014). In this organism, the formation of the translationally inactive 100S ribosomes is mediated by the sequential action of a ribosomal modulation factor (RMF) and a hibernation promoting factor (HPF) (Wada *et al.*, 1995). The dimerization reaction is reversible, as the 100S ribosomes dissociate back into 70S ribosomes within minutes after the cells are transferred into fresh medium. 100S formation may help preserve the ribosomes in non-growing cells, and consistent with this assumption, an *rmf* deletion mutant exhibited reduced survival upon prolonged storage (Yamagishi *et al.*, 1993). Characterization of 100S formation in other bacteria indicates that the paradigm for RMF/HPF-mediated formation of 100S ribosomes established in *E. coli* is restricted to Gammaproteobacteria, as RMF homologues are only present in this group of bacteria. However, HPF homologues are conserved in almost all bacteria, as well as in plant plastids. In contrast to Gammaproteobacteria, the HPF homologue in other bacteria has a long tail region at the C-terminus (Ueta *et al.*, 2008, 2013). These long HPFs appear to have a conserved role in ribosomal dimerization as long HPF homologues from diverse bacteria such as *Thermus thermophilus*, *L. lactis*, *Lactobacillus paracasei*, *Listeria monocytogenes* and *Staphylococcus aureus* are capable of mediating 100S ribosomal formation (Kline *et al.*, 2015; Puri *et al.*, 2014; Ueta *et al.*, 2010, 2013). In *E. coli*, the formation of 100S ribosomes is associated with the entry into stationary phase and 100S dimers are not detected in rapidly growing cells in this bacterium. In contrast, 100S ribosomes are abundant throughout the growth curve of the two Gram-positive bacteria, *S. aureus* and *Listeria monocytogenes* (in exponential cells, the ratio of 70S to 100S is in fact close to 1 : 1) (Kline *et al.*, 2015; Ueta *et al.*, 2010). This observation opens up the interesting possibility that the formation of 100S ribosomes is induced by alternative signals and serves alternative roles in bacteria other than the Gammaproteobacteria.

In this study, *L. lactis* cells were exposed to glucose starvation during batch fermentation in chemically defined Synthetic Amino acid (SA) medium (Jensen & Hammer, 1993). Exponential growth for at least eight generations was ensured by serial dilution of the culture in media containing less than 1 % glucose. Under these conditions, the strain grows at its maximal growth rate until growth arrests abruptly as a result of glucose depletion. Protein expression following glucose depletion was visualized by separating [^{35}S]methionine-labelled proteins by 2D-PAGE. This analysis revealed that while protein synthesis stopped almost completely following glucose starvation, a few selected proteins escaped the translational arrest and were still being produced. Interestingly, Yfia, a protein shown to be required for ribosomal dimerization in *L. lactis* (Puri *et al.*, 2014), was among the most highly produced proteins following glucose depletion.

METHODS

Media and growth conditions. The *L. lactis* strains were grown in either M17 media (Terzaghi & Sandine, 1975) supplemented with 1 % glucose (GM17) or the chemically defined medium SA (Jensen & Hammer, 1993) supplemented with 1 % glucose (GSA). During glucose starvation experiments, lactococcal strains were grown in SA medium with glucose added as indicated and were incubated at 30 °C unless otherwise stated. *E. coli* DH5α [Δ(*lacZYA-argF*) Φ80*lacZ*Δ*M15 recA1 endA1hsdR17*] cells were grown in lysogeny broth at 37 °C. Antibiotics were added when required in the following concentrations: ampicillin, 100 µg ml⁻¹ for *E. coli*; erythromycin, 2 µg ml⁻¹ for *L. lactis* and 150 µg ml⁻¹ for *E. coli* and tetracycline, 2 µg ml⁻¹ for *L. lactis*.

To ensure balanced growth until glucose depletion, a pre-culture in SA, pre-warmed to 30 °C, was inoculated with an exponentially growing overnight culture in GSA with an OD_{450} not exceeding 0.4, resulting in an OD_{450} of 0.05. The pre-culture with 0.06 % glucose was grown to an OD_{450} of 0.4 and was used to inoculate a final culture (0.02 % glucose) to an OD_{450} of 0.05, again in SA at 30 °C. This scheme leads to a depletion of glucose at an OD_{450} of 0.2–0.4, depending on the inoculum volume. To obtain a more defined start point of energy starvation, 2-deoxy-glucose was added to a culture in SA medium containing 0.06 % glucose to a final concentration of 100 mM.

Survival and revival during long-term glucose starvation. To perform long-term starvation experiments (analysis of survival), glucose starvation was initiated as previously described, and after inoculating the cells into the final medium with 0.02 % of glucose, the cultures were aliquoted into 10 ml plastic tubes and placed at 30 °C. To monitor survival during the course of the experiment, tubes were then withdrawn at various time points for optical density measurement and determination of viable counts by plating on GM17 plates after dilution in 0.9 % NaCl.

To monitor the ability of the starved cells to resume growth when re-supplemented with glucose (revival), 300 µl of the starved cultures were transferred in triplicate to the wells in an automated Bioscreen incubator (Microbiology Reader BioScreen C from Oy Growth Curves AB). Glucose was added to two of the wells, resulting in a glucose concentration of 0.4 %, and growth at 30 °C was monitored by measuring the OD_{450} in the BioScreen. To obtain a corrected growth curve during revival, the data had to be corrected not only for the optical density of medium but also for the dead cells, since they constituted an increasing proportion of the cells as the starvation experiment progressed. This correction was obtained by subtracting the optical density of the well without glucose from the optical density of the wells with glucose. However, since this also leads to the subtraction of the remaining living cells, the optical density of these cells was added. The optical density of the living cells was calculated from the c.f.u. ml⁻¹ obtained from the plating of the starved culture.

DNA techniques and transformation. DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989). Enzymes and corresponding buffers were obtained from Amersham Pharmacia Biotech or MBI Fermentas. The Ampli*Taq* DNA polymerase was used for PCR amplifications (Perkin Elmer Cetus), and primers were obtained from TAG Copenhagen, Denmark. DNA sequencing was performed by MWG.

Lysing of the lactococcal cells prior to isolation of chromosomal DNA was obtained by first freezing for 30 min at $-80\,^{\circ}$C then thawing to room temperature and finally treating with lysozyme at a final concentration of 20 µg l^{-1} for 30 min at 37 $^{\circ}$C, after which DNA was prepared as described for *E. coli* (Sambrook *et al.*, 1989). Plasmid DNA was isolated from both *E. coli* and lactococcal cells by the alkaline lysis technique (Sambrook *et al.*, 1989) and was applied on Qiagen DNA purification columns when a higher purity of DNA was required. To ensure lysis of the lactococcal cells before plasmid preparation, cells were treated with lysozyme at a final concentration of 20 µg l^{-1} for 20 min at 37 $^{\circ}$C.

For transformation, *E. coli* that are CaCl$_2$ competent were prepared and used as previously described (Sambrook *et al.*, 1989), whereas *L. lactis* were made competent and transformed by electroporation as previously described (Holo & Nes, 1989).

Construction of strains. The construction of the *yfiA* deletion strain AB25 was initiated by PCR amplification of a fragment from *L. lactis* MG1363 (Gasson, 1983) chromosomal DNA upstream of *yfiA* using the primers p555-6 (GGATTGCATCGGTCACTTCG) and p555-7 (GGGGAAGCTTGACGTTTTCAATATCATG). A downstream fragment was obtained in a similar manner using primers p555-3 (GGGGGGGATCCGTCGTACAGATGGTCG) and peno-2 (CCCCTC TAGACCACCGTTGATGATGTTC). The two fragments were digested with *Bcl*I and *Bam*HI, respectively, and ligated. The ligation mix was used as template in a third PCR reaction with the primers p555-4 (GGGGAAGCTTCAAACTTGGGTGCAACG) and peno-2. The resulting fragment of 1.3 kb was inserted in the pMOS*Blue* (Amersham) vector according to the procedure proposed by the manufacturer, resulting in plasmid pAB21.

The insert in pAB21 was moved after digestion with *Pst*I and *Spe*I into plasmid pORI28 (Leenhouts *et al.*, 1996) digested with *Pst*I and *Xba*I. The resulting plasmid was named pAB24. An MG1363 derivative with pAB24 inserted in the chromosome through homologous recombination in the downstream *yfiA* fragment was subsequently obtained after transformation with pAB24 and termed AB24-1.

In order to obtain the deletion of the *yfiA* gene, the integrated plasmid in AB24-1 needed to cross out from the chromosome, using the upstream fragment. This process was promoted by introducing pG$^+$host8 (Maguin *et al.*, 1996) in the strain, from which a functional RepA is produced at 28 $^{\circ}$C. At higher temperatures, the RepA protein is inactive. The presence of RepA at 28 $^{\circ}$C thus leads to the initiation of replication from the pORI replicon in pAB24, promoting recombination (Law *et al.*, 1995; Leenhouts *et al.*, 1996). Subsequently, penicillin counterselection against erythromycin resistance was carried out at high temperature to further enrich for the strains in which crossing out had been successful. The cells were subjected to growth in GM17 in the presence of erythromycin (2 µg ml^{-1}) for hours before ampicillin was added to 100 µg ml^{-1}. After 2 h, cells were harvested by centrifugation and plated on GM17. After growth overnight, colonies were screened for erythromycin sensitivity. By this procedure, two Erm^S strains were obtained, which were demonstrated by PCR on chromosomal DNA by primers p555-9 (GGGGGGATCCAAAAGTTTAAGGACTTC) and peno-1 (GGGGTCTAGATGTTGGGTTACCGCGTGAGTC) to contain the desired deletion. The DNA sequence of the deletion point was verified, and one of the strains was termed AB25 and used further in this study.

Strain AMB2016 was constructed by insertion of plasmid pAMB2016 carrying *yfiA* into the bacteriophage TP901-1 chromosomal attachment site of AB25. For the transformation, pLB95 encoding the phage integrase was included in a co-transformation with pAMB1966 (Brøndsted & Hammer, 1999). The plasmid was constructed by cloning of a *yfiA* harbouring PCR fragment into the *Xho*I and *Pst*I restriction enzyme sites of plasmid pLB86 (Brøndsted & Hammer, 1999). The primers used to amplify the fragment from *L. lactis* MG1363 were (MK772:

CAGACCCTCGAGCATATTTCCATTGGTGCTTC) and (MK773: CAGACCCTCGCAGCCTTAATTTTTATTCTGTTTCAATTAAGC).

Two-dimensional gel electrophoresis and identification of proteins. Proteins were labelled with [^{35}S]methionine in SA medium, analysed by 2D gel electrophoresis, and selected spots were identified by automated Edman degradation as previously described (Kilstrup *et al.*, 1997).

Microarray analysis. RNA was extracted from balanced cultures growing under glucose excess and from cultures exposed to abrupt glucose depletion. Total RNA isolation was performed as described previously (Jendresen *et al.*, 2014) ensuring efficient quenching by rapid cooling. The RNA was analysed using Agilent custom microarrays mapped to the *L. lactis* subsp. *cremoris* MG1363 genome (RefSeq: NC_009004.1) with five probes per gene. Images were analysed with an Agilent 2-micron scanner, and numerical values for features were calculated from the images with Agilent Feature Extraction software. The raw probe level data were quantile normalized after background subtraction using the 'Limma' package (Smyth, 2005) under Bioconductor (Gentleman *et al.*, 2004). Quantification of differential expression was subsequently performed using 'basicRMA' from the 'Oligo' package (Carvalho & Irizarry, 2010) under Bioconductor. The microarray data are available at GEO with the accession number GSE78882.

Metabolomics. The nucleotide pool sizes were determined by the liquid chromatography mass spectrometry method essentially as described by Magdenoska and co-workers (Magdenoska *et al.*, 2013), except that 1.5 ml culture was added to a pre-chilled 2 ml Eppendorf tube with 0.3 ml of 10 M HCOOH and 19.5 µl of ^{13}C internal standard solution. The quenching method was previously shown to efficiently quench metabolism allowing an accurate determination of nucleotide pools (Martinussen *et al.*, 2003).

RESULTS

## Few proteins escape translational arrest following abrupt glucose depletion

To investigate the effect of glucose starvation on protein synthesis, a culture of *L. lactis* subsp. *cremoris* MG1363 was grown exponentially for more than eight generations in SA medium with less than 1 % glucose as described in Methods. Under these conditions, the strain grows at its maximal growth rate until growth arrests abruptly at a reproducible cell density of OD$_{450}$ 0.4 (Fig. 1a). Immediately after the growth arrest, a small aliquot of the culture was transferred to pre-warmed Eppendorf tubes containing [^{35}S]methionine for labelling of newly formed proteins. Initially, proteins were labelled for 10 min, but when the proteins were analysed by 2D gel electrophoresis and autoradiography, no labelled proteins could be detected (data not shown). In contrast, the normal protein synthesis pattern could be identified when the strain was grown in excess of glucose (Fig. 1b), showing that protein synthesis was severely reduced in the glucose-starved cells. In order to increase the sensitivity of the detection, the time for [^{35}S]methionine incorporation was extended to 60 min following glucose depletion. Using this procedure, a few labelled proteins could be identified (Fig. 1c), including one protein of approximately 25 kDa that was synthesized at a rate higher than that of the others. Identical protein profiles were

observed if the proteins were labelled for 2 h or if the 1 h labelling was postponed for 1 h relative to the onset of starvation (data not shown). Addition of 2-deoxy-glucose is an alternative way to induce glucose starvation and energy depletion, as 2-deoxy-glucose is phosphorylated to the non-metabolizable 2-deoxy-glucose-6-phosphate via the PTS transport system and competitively inhibits the uptake and metabolism of glucose (Kornberg & Lambourne, 1994). Similar to glucose depletion, addition of 2-deoxy-glucose to exponentially growing cultures of *L. lactis* MG1363 resulted in abrupt growth arrest (data not shown) and proteomic analysis of 2-deoxy-glucose-treated cells revealed a proteomic profile very similar to Fig. 1c (data not shown). Taken together, these data suggest that while general protein synthesis is arrested in response to glucose depletion, the cells prioritize to synthesize a very limited number of proteins, with one protein in particular.

Depletion of glucose results in an extremely low GTP concentration

We suspected that the low translational efficiency could be due to energy depletion. Therefore, we measured the intracellular concentrations of nucleotide metabolites before and after the onset of glucose starvation. Fig. 2 shows that the energy charge, defined as $[(ATP+0.5\times ADP)/(ATP+ADP+AMP)]$ (Atkinson, 1968), decreases abruptly during
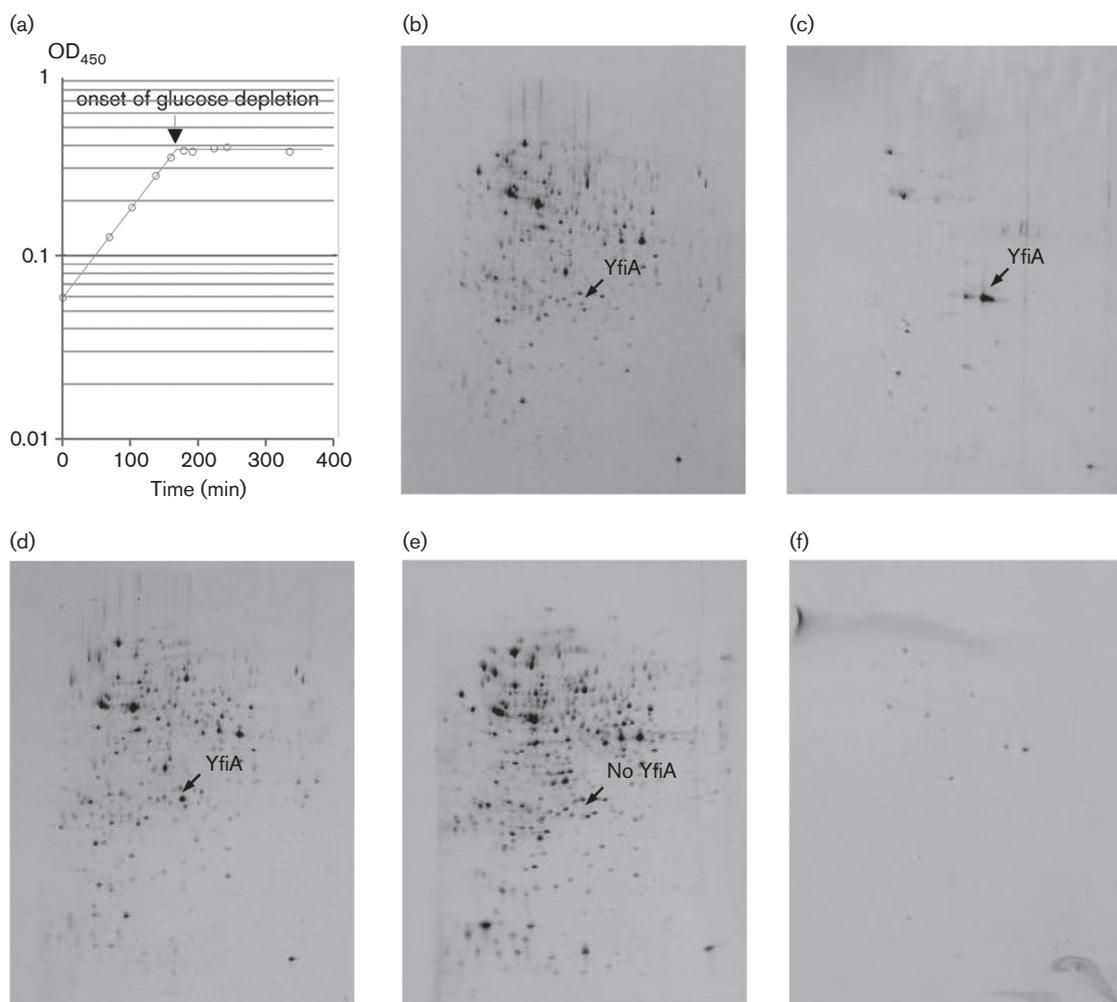
Fig. 1. Proteome of *L. lactis* under glucose depletion. (a) Growth curve of MG1363 in SA medium with a limited supply of glucose, monitored by the OD_{450} of the culture. An arrow indicates the time at which glucose runs out. (b–f) Autoradiograms of [35S]methionine-labelled protein extracts obtained from *L. lactis* strains MG1363 (WT, b–d) or AB25 (ΔyfiA, e and f) grown in SA medium with excess or limiting glucose and separated by 2D-PAGE. (b) Proteome of MG1363 grown in excess of glucose and labelled for 10 min at OD_{450} 0.4. (c) Proteome of MG1363 grown in glucose-limited medium, labelled for 1 h immediately following glucose depletion. (d) Identification of the location of YfiA in the MG1363 proteome. A mixture of the labelled extracts shown in (b) and (c) was analysed by 2D-PAGE. (e) Proteome of AB25 grown in excess of glucose and labelled for 10 min at OD_{450} 0.4. (f) Proteome of AB25 grown in glucose-limiting medium, labelled for 1 h immediately following glucose depletion. Location of YfiA is indicated from (b) to (e).
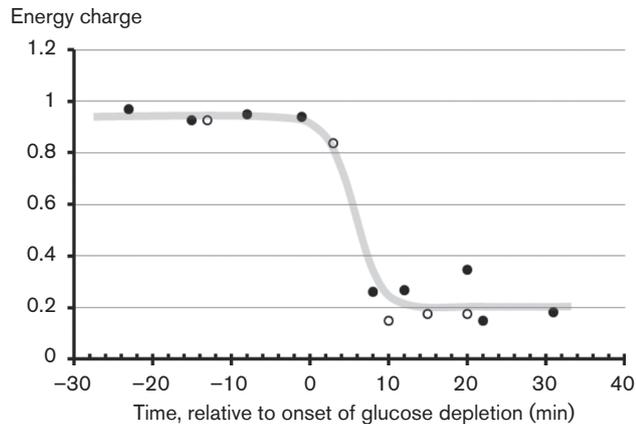
Energy charge

Fig. 2. Energy charge of cultures during glucose depletion. The energy charge is calculated from the nucleotide pool sizes by the equation $(ATP + \frac{1}{2}ADP)/(ATP + ADP + AMP)$. Filled symbols, MG1363; open symbols, AB25.

glucose depletion to extremely low levels, showing that the cells' energy level was severely exhausted.

ATP, GTP, UTP and CTP pools all decreased after glucose depletion, but following different kinetics. ATP and UTP decreased slightly in the last 30 min before glucose was used up (Fig. 3a and c), suggesting that the glucose metabolism was somewhat lowered in this phase even though the growth rate was unaffected. Just before glucose was totally depleted, cellular levels of ATP and UTP dropped rapidly to around 20 % of their pre-shift values. The levels of GTP and CTP appeared to accumulate more than twofold in the same short period but then dropped to extremely low levels. Mass balance analysis of nucleotide monophosphate, diphosphate and triphosphate levels (Figs S1 and S2, available in the online Supplementary Material) was consistent with constant A and T nucleotide levels, while a net input of G and C nucleotides was required to account for the rise in GTP and CTP levels around the time of glucose depletion. This will be discussed later.

The transcriptomic response to abrupt glucose depletion overlaps with the CcpA regulon

To gain insight into the mechanism underlying the abrupt arrest in protein synthesis, the cellular response of *L. lactis* towards glucose exhaustion was analysed by comparing the transcriptome before depletion to the transcriptome 10 and 60 min after glucose depletion by DNA microarray analysis. From the fluorescence distribution profile (Fig. S3), it was clear that the mRNA levels were not lowered to a degree where they could be the prime cause of the translational arrest. The highest peak of the profile was shifted slightly towards a lower level, showing that the levels of the most abundant RNA species were diminished, while the overall profile was maintained. A constant profile during glucose depletion could arise from one of two extreme situations,

(i) either both transcription and RNA degradation proceeded at normal speed or (ii) where both were arrested to the same degree. Since we found that the GTP and CTP concentrations were lowered to almost undetectable levels and that the ATP and UTP concentrations were severely lowered after 5 min, the latter explanation is most plausible. Thus, we assume that the transcriptional regulatory machinery was able to respond to glucose depletion for 5 min until total GTP depletion and the transcriptome was 'frozen' in this state by reduced mRNA degradation (Redon *et al.*, 2005a).

The composition of mRNA levels in the transcriptome was clearly rearranged 10 min after glucose depletion when compared to the pre-stress levels (see Tables S1–S6 for details). The profile showed differential regulation of genes from several regulons that might be expected for a glucose downshift. Many genes involved in sugar metabolism from the CcpA (Zomer *et al.*, 2007) and the FruR (Barrière *et al.*, 2005) regulon, as well as genes from the arginine-responsive ArgA/AhrC (Larsen *et al.*, 2008) regulons were affected.

The major glucose starvation protein is the *L. lactis* homologue of the ribosomal dimerization factor YfiA

To identify the major protein synthesized following glucose depletion, the protein preparations shown in Fig. 1(b) and (c) were mixed and then applied on a new 2D gel (Fig. 1d). The major glucose starvation protein could now be identified at a position corresponding to a molecular mass of ~25 kDa and a pI of ~5.7. When inspecting the same position on the 2D gel with proteins from cells grown under glucose excess, a weak spot could be identified at this position (Fig. 1b), showing that the protein is also synthesized in the presence of glucose. The N-terminal amino acid sequence of the 25 kDa protein was determined by automated Edman degradation to be X-I-X-F-N-I-R-L/G-E-N-I/V. A perfect match was found to the N-terminal amino acids (M-I-K-F-N-I-R-G-E-N-V) of the gene product from the *llmg_0616* gene, annotated as YfiA, a protein recently shown to belong to the family of long hibernation promotion factors and to be necessary and sufficient for ribosomal dimerization in *L. lactis* (Puri *et al.*, 2014).

To confirm that the 25 kDa protein was identical to the gene product of the *yfiA* gene and to permit analysis of the function of the protein, we introduced an in-frame deletion in *yfiA* in MG1363, resulting in mutant strain AB25. 2D-PAGE analysis clearly demonstrated that AB25 does not produce YfiA (Fig. 1e), confirming that the 25 kDa protein and YfiA are the same protein.

Except for the lack of YfiA, the protein expression pattern appears virtually identical in MG1363 and AB25 under non-stressed conditions, demonstrating that YfiA is not required for global gene regulation under exponential growth [comparison of Fig. 1(b) and (e) taking into account that a higher specific activity of labelled methionine was used in Fig. (1e)].

(a)

ATP

(b)

GTP

(c)

UTP

(d)

CTP
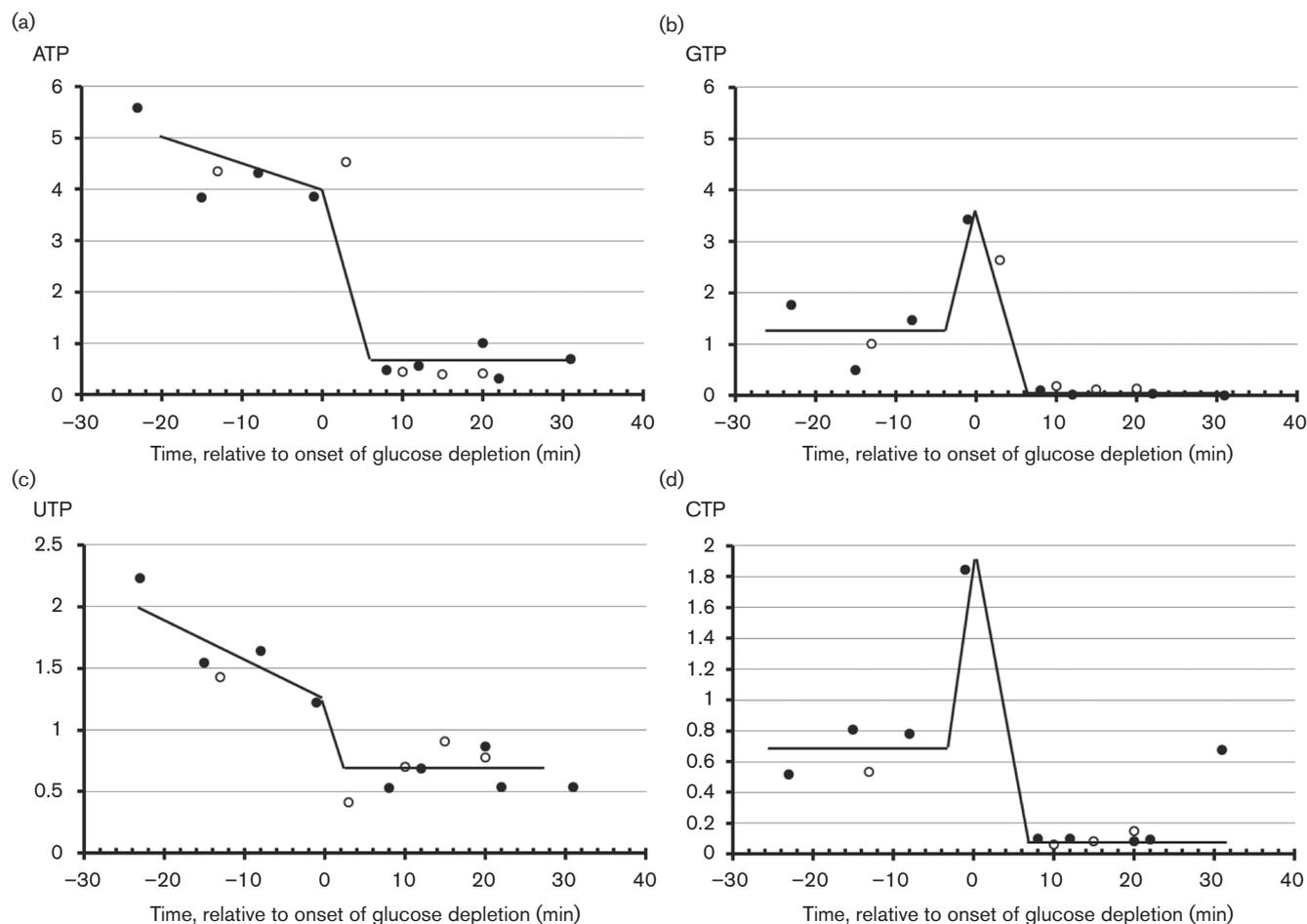
Fig. 3. Intracellular NTP levels during glucose depletion. Filled symbols: metabolite levels from three biological replicate cultures of MG1363. Open symbols: metabolite levels from three biological replicate cultures of AB25. One point represents the determination of a single sample originating from one of the six independent cultures. (a) ATP; (b) GTP; (c) UTP; (d) CTP. Lines are tentative. The nucleotide concentration on the y-axis is in nmol mg^{-1} dry weight.

When AB25 was subjected to glucose starvation, 2D-PAGE analysis of S-methionine-labelled proteins (1 h of labelling) revealed the absence of the major glucose starvation protein spot, again confirming that YfiA is the major protein synthesized in glucose-depleted cells (Fig. 1f). Since YfiA is responsible for ribosomal inactivation via dimerization (Wada *et al.*, 1995), it could be speculated that YfiA was responsible for the arrest of protein synthesis observed in glucose-starved cells. However, the observation that arrest of protein synthesis n response to glucose starvation is also observed in the *yfiA* deletion mutant conclusively shows that this is not the case.

YfiA is required for survival during prolonged glucose starvation

Since YfiA is the main protein synthesized under glucose starvation, we investigated whether deletion of the gene led to an altered physiology and response to glucose starvation. We found that the growth rate of AB25 in GSA medium

was 17 % lower than that of MG1363 (data not shown). Even though the WT and mutant strains grew with different growth rates, a similar abrupt growth arrest was encountered at approximately the same cell density with AB25 as with MG1363 (data not shown). Thus, YfiA does not seem to be required for the immediate growth arrest in response to glucose depletion. Changes in nucleotide pools were also identical with the WT (Figs 2 and 3).

However, a phenotype of the *yfiA* deletion was observed when survival of the *yfiA* mutant strain during long-term glucose starvation in liquid SA medium was compared to the survival of MG1363. Starvation experiments with both the WT and *yfiA* deleted strain were performed as described above, but now the glucose-depleted cultures were left at 30 °C for up to 23 days. Survival was monitored frequently by c.f.u. determination on GM17 plates. The graph in Fig. 4 shows the average survival and their variance from four such independent experiments. It appears that the death curve has three phases in the semi-logarithmic plot. During

the first 5 days, the death rate appears to be similar for MG1363 and the *yfiA* mutant, but the curve is more complex than the curve for a simple exponential decay. In Fig. 4, the logarithmic decay has been fitted to a parabolic function. Between 5 and 15 days of starvation, the death rate of the two cultures appears to be exponential with equal half-life, as the fraction of surviving cells follows straight lines with the same slope in the semi-logarithmic plot.

To show that *yfiA* is responsible for survival during prolonged glucose starvation, a WT *yfiA* allele was integrated on the chromosome at the bacteriophage TP901-1 attachment site in AB25, thus obtaining AMB2016. After 10 days of starvation, the survival of AB25 ($\triangle yfiA$) was five times lower than both the WT and AMB2016 complementation strain. After 29 days of starvation, the survival rate of AB25 was determined to $5 \times 10^{-6} \pm 3 \times 10^{-6}$. For the WT, the survival rate was 1 to 2 orders of magnitudes higher ($150 \times 10^{-6} \pm 20 \times 10^{-6}$). Intriguingly, the complementation strain behaved as the WT with respect to survival rate ($370 \times 10^{-6} \pm 330 \times 10^{-6}$). This experiment clearly shows that *yfiA* is required for optimal survival during prolonged glucose starvation.

### YfiA promotes re-growth after glucose starvation

During the long-term starvation experiments, we observed that plating of the starved WT cells resulted in markedly bigger colonies after 1 day of incubation than did plating of the starved *yfiA* mutant cells and that the size of the mutant colonies decreased with increasing starvation time (data not shown).

To investigate this phenomenon in more detail, we assessed the ability of the starved cultures to resume growth by adding back glucose to the starved cultures at various time points following glucose depletion and monitoring growth in an automated BioScreen incubator. From the growth curves, we could observe an apparent lag phase before the optical density of the culture increased. Fig. 5a shows three such growth curves of MG1363 after addition of glucose to cultures that have been starving for 2, 10 and 23 days, respectively. While the apparent lag phase increased in relation to the starvation time, this difference vanished when the growth curves were extrapolated (slanted broken lines) back to the optical density of the surviving cells at the time of glucose addition (shown as horizontal broken lines). When this extrapolation was made, MG1363 appeared to start re-growth within the first hours after glucose addition during all 23 days of glucose starvation (Fig. 5b). In contrast, the *yfiA* mutant appeared to require a lag phase of more than 5 h in order to start re-growth after 15 days of starvation, supporting the assumption that YfiA is important for keeping the cells in a growth-competent state.

DISCUSSION

Following abrupt glucose depletion, obtained after several generations of exponential growth in GSA medium at reduced glucose levels, we found that the cell entered a
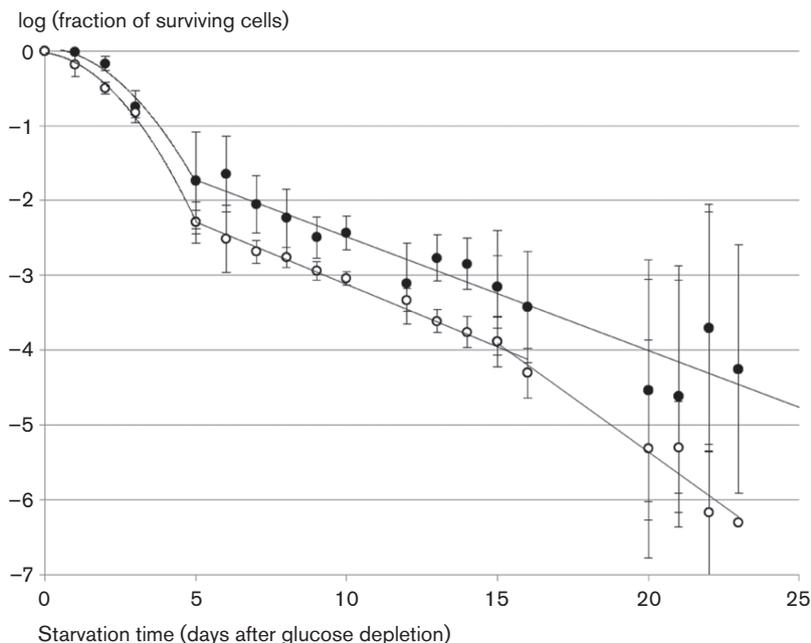
Fig. 4. Survival after abrupt glucose depletion. MG1363 (filled circles) and AB25 (open circles) were exposed to glucose starvation as described in Methods, and survival was monitored for the next 23 days by diluting and plating. Survival was calculated relative to the number of cells in the culture at day 0, and the graph shows the means of the log of the relative survival for the two strains obtained in four independent experiments.

physiological state of low energy charge and with an extremely low GTP concentration, in which translation was almost completely abandoned, while mRNA levels were largely unaltered. Transcription elongation has been found preferentially to require high UTP and ATP concentrations because of their high K_M. The K_M constants for the *Salmonella typhimurium* RNA polymerase were found to be 0.6, 0.9, 0.4 and 0.1 mM for ATP, UTP, GTP and CTP, respectively (Jensen *et al.*, 1986). Translation on the other hand is mostly dependent upon the GTP concentration as the ribosomal elongation is energized by GTP hydrolysis by elongation factors EF-G and EF-Tu. In *E. coli*, the K_M (GTP) values were found to be around 0.5 μM, much lower than for the RNA polymerase (Mesters *et al.*, 1994).

A hypothetical scenario could account for the nucleotide changes in *L. lactis* if the kinetic values were approximately correct. Approaching glucose depletion, the gradual lowering of the UTP and ATP concentrations to below their K_M values might have slowed the mRNA synthesis and resulted in accumulation of GTP and CTP, which had not yet reached their K_M. At the point of depletion, the low K_M (GTP) of the elongation factors could allow the ribosomes to proceed for a few minutes until the GTP concentration decreased to below this concentration. Since CTP synthesis from UTP by the CTP synthase is totally dependent upon GTP activation (Willemoës *et al.*, 2005), the severe drop in CTP was as expected.
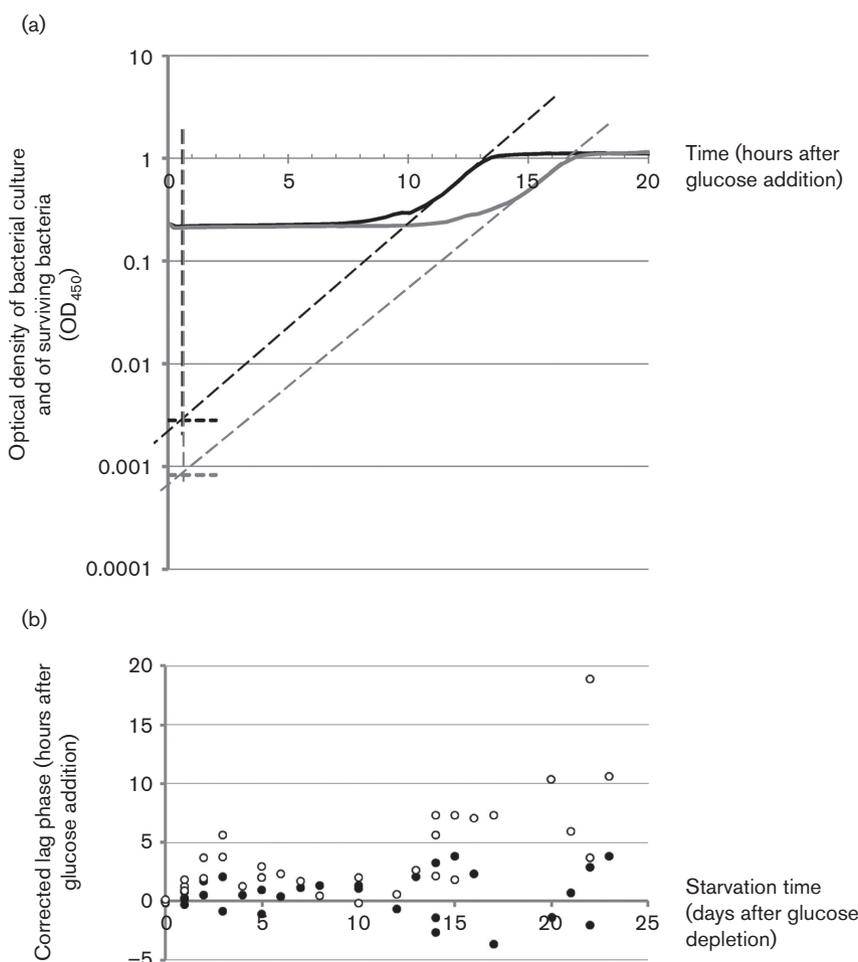

Fig. 5. Lag phase after replenishment of glucose to starved culture. (a) Principle for calculating the true length of the lag phase after glucose starvation. Cultures of *L. lactis* MG1363 were subjected to glucose starvation for 10 and 23 days, followed by a supplement of glucose at time zero. Growth at 30 °C was monitored by measuring the OD_{450} in an automated Bio-Screen incubator. The continuous lines indicate the optical densities from the BioScreen incubator. The growth curves after replenishment of glucose after 10 and 23 days starvation are shown as black and grey lines, respectively. By extrapolation of the growth curves (slanted broken lines) to the calculated initial optical density of the surviving cells obtained from Fig. 4 (horizontal broken lines), a corrected duration of the lag period could be estimated (vertical broken lines). (b) Corrected lag phases in hours from a large number of revival experiments (as shown in a) are shown as a function of the duration of the glucose starvation for MG1363 (filled circles) and AB25 (open circles).

In a previous study, mRNA half-life was found to increase more than threefold upon glucose starvation in a pH-controlled chemically defined medium (Redon *et al.*, 2005a). The authors concluded that the changes in mRNA levels under glucose starvation were determined by both altered transcription and altered degradation (Redon *et al.*, 2005b). This could account for the constant mRNA level 10 min after glucose depletion in the present study.

Amazingly, the synthesis of a few proteins escaped the severe translational arrest following glucose depletion by an unknown mechanism. YfiA, the ribosomal dimerization factor of *L. lactis* (Puri *et al.*, 2014), was synthesized at the highest rate, as visualized by incorporation of radioactive methionine in nascent proteins and separation by 2D gel electrophoresis. YfiA is also synthesized in the non-stressed cells, and in fact, the synthesis rate seems to be similar in starved and non-starved cells, as the relative intensity of the YfiA spots synthesized before (Fig. 1b) and after glucose depletion (Fig. 1c) is proportional to the labelling time. The continued synthesis of the YfiA protein suggested that YfiA activity is important in the response towards energy deprivation, which is consistent with previous findings showing that an *L. lactis yfiA* mutant exhibited reduced viability after long-term starvation compared to the MG1363 WT (Puri *et al.*, 2014). Ribosomal dimerization was proposed to be a mechanism to regulate protein synthesis capacity (Puri *et al.*, 2014), and we first speculated that YfiA binding to the ribosome is responsible for observed inhibition of translation. Biologically, it makes sense for the cells to overexpress a ribosomal dimerization factor during low translational activity in order to store the inactive ribosomes. However, since translation ceased also in a *yfiA* deletion mutant, the translational arrest following glucose depletion does not seem to be dependent on YfiA and ribosomal dimerization.

At present, we do not know the mechanism allowing preferential synthesis of YfiA in glucose-depleted cells. Interestingly, a homologue of YfiA encoded by the *lrtA* gene (light repressed transcript) is one of a few proteins expressed, when the cyanobacterium *Synechococcus* PCC7002 is suddenly deprived of light resulting in energy depletion (Samartzidou & Widger, 1998). The continued synthesis of LtrA coincided with a pronounced stabilization of the *ltrA* transcript, and the capability of the *ltrA* leader sequence to fold into an extended secondary structure was hypothesized to account for this stabilization of the *ltrA* mRNA (Samartzidou & Widger, 1998). Analysis of the *yfiA* sequence predicts that the *yfiA* leader mRNA similarly has the potential to fold into an extended secondary structure (Fig. 6) that might contribute to the continued synthesis of YfiA, either by stabilizing the *yfiA* transcript or by facilitating selective translation of the *yfiA* mRNA. Such a mechanism has been suggested to account for the preferential translation of cold shock-induced mRNAs in *E. coli* cells shifted to lower temperatures (Gualerzi *et al.*, 2003). Interestingly, the Shine–Dalgarno is potentially sequestered in a stem–loop structure that might prevent translation during normal growth. How YfiA is translated after glucose depletion is not obvious.
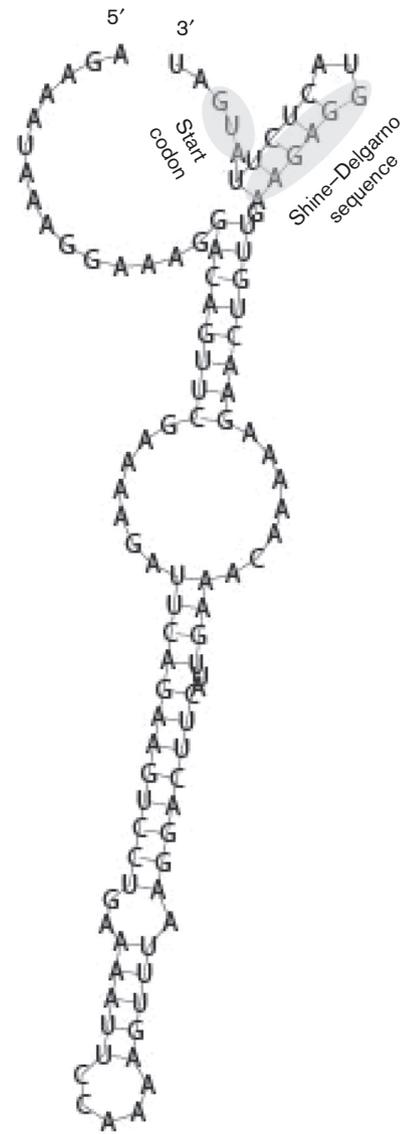
Fig. 6. Potential secondary structure of the *yfiA* leader. The potential Shine–Dalgarno sequence and start codon is shown. The folding was performed and downloaded from the RNAfold Web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) at the Institute for Theoretical Chemistry, University of Vienna.

Similarly to Puri *et al.* (2014), we found that YfiA is important for survival during long-term starvation and for keeping the long-term starved cells in a growth-competent state. Strikingly, after several weeks of starvation, wildtype cells were still able to initiate growth almost instantaneously after the addition of glucose, while a lag phase was found for the *yfiA* mutant, which increased proportional to the duration of the energy deprivation. Given the role of YfiA in ribosomal dimerization, these data strongly indicate that YfiA-mediated dimerization is important for preserving the ribosomes in a state that allow rapid re-growth of long-term starved cells. The starvation-specific phenotypes of

our mutant are very similar to the reported phenotypes of the previously characterized *yfiA* mutant (Puri *et al.*, 2014). However, while Puri *et al.* (2014) observed that their *yfiA* deletion in a similar experimental setup was incapable of re-initiating growth after 16 days of starvation, our *yfiA* mutant remained growth competent up to the end of the experiment (23 days).

Additionally, we found that deletion of *yfiA* reduced the exponential growth rate, suggesting that YfiA activity is important also for growing *L. lactis* cells. In line with this observation, we found that YfiA is synthesized in exponential cultures (Fig. 1b). In contrast, the *yfiA* deletion mutant characterized by Puri *et al.* (2014) did not reduce growth rates despite that their strain similar to ours was derived from *L. lactis* MG1363. We tentatively attribute this difference to deviations in the experimental setups, such as the use of different media compositions.

To our knowledge, *L. lactis* is the only organism in which stress-related phenotypes conferred by inactivation of the ribosomal hibernation factor have been reported. Lactococcal *yfiA* is identical with 'orf55' that was reported to be the last gene in an operon also encompassing the classical heat shock genes, *ctsR* and *clpC*, suggesting that transcription of *yfiA* is induced by heat stress and other stresses (Varmanen *et al.*, 2000). Consistent with this prediction, a number of studies have shown that synthesis of ribosomal hibernation factors is generally stress inducible: in *Listeria*, transcription of the *hpf* gene, encoding the ribosomal dimerization factor, is strongly up-regulated in response to carbon starvation and exposure to salt, ethanol and heat stress, all of which were abrogated in the absence of the stress-responsive sigma factor σ^B (Kline *et al.*, 2015). Similarly, the *S. aureus* Hpf homologue (SACOL0815) was one of only two proteins induced by three different types of oxidative stress; additionally, transcription of the gene was reported to be inducible by heat stress and by addition of mupirocin, an inducer of the stringent response (Anderson *et al.*, 2006; Reiss *et al.*, 2012; Wolf *et al.*, 2008). Taken together, these studies support that ribosomal dimerization has a general role in the adaptation to stresses in *L. lactis* and other Firmicutes.

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