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Protein costs do not explain evolution of metabolic strategies and regulation of ribosomal content: does protein investment explain an anaerobic bacterial Crabtree effect?

Anisha Goel,1,2,3† Thomas H. Eckhardt,4† Pranav Puri,5† Anne de Jong,4 Filipe Branco dos Santos,1,3 Martin Giera,1 Fabrizia Fusetti,5 Willem M. de Vos,2 Jan Kok,3,4 Bert Poolman,1,3,5 Douwe Molenaar,1,3 Martin Giera,1 Fabrizia Fusetti,5 Willem M. de Vos,2 Jan Kok,3,4 Bert Poolman,1,3,5 Douwe Molenaar,1,3 Oscar P. Kuipers3,4,* and Bas Teusink1,3,*
1 Systems Bioinformatics IBIVU, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands.
2 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
3 Kluyver Centre for Genomics of Industrial Fermentation/Netherlands consortium for Systems Biology (NCSB), P.O. Box 5057, 2600 GA Delft, The Netherlands.
4 Departments of Genes and Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Netherlands Proteomics Centre and Zernike Institute for Advanced Materials (ZIAM), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands.

Summary

Protein investment costs are considered a major driver for the choice of alternative metabolic strategies. We tested this premise in Lactococcus lactis, a bacterium that exhibits a distinct, anaerobic version of the bacterial Crabtree/Warburg effect; with increasing growth rates it shifts from a high yield metabolic mode [mixed-acid fermentation; 3 adenosine triphosphate (ATP) per glucose] to a low yield metabolic mode (homolactic fermentation; 2 ATP per glucose). We studied growth rate-dependent relative transcription and protein ratios, enzyme activities, and fluxes of L. lactis in glucose-limited chemostats, providing a high-quality and comprehensive data set. A three- to fourfold higher growth rate rerouted metabolism from acetate to lactate as the main fermentation product. However, we observed hardly any changes in transcription, protein levels and enzyme activities. Even levels of ribosomal proteins, constituting a major investment in cellular machinery, changed only slightly. Thus, contrary to the original hypothesis, central metabolism in this organism appears to be hardly regulated at the level of gene expression, but rather at the metabolic level. We conclude that L. lactis is either poorly adapted to growth at low and constant glucose concentrations, or that protein costs play a less important role in fitness than hitherto assumed.

Introduction

Cells have the remarkable ability to adapt to their environment, and this invokes two complementary questions: how do they manage to adapt and why is the underlying mechanism or strategy successful in the survival of the fittest? In this paper, we study the adaptation to glucose availability, which in many organisms results in a seemingly paradoxical metabolic reprogramming called the (bacterial) Crabtree effect in microorganisms or the Warburg effect in cancer cells (Warburg, 1956; DeBerardinis and Thompson, 2012). At increasing glucose levels – or glycolytic flux, or growth rate, this is still open for debate – cells switch from a high-yield strategy, such as respiration, to a metabolic strategy that generates less adenosine triphosphate (ATP) per glucose, i.e. fermentation. Such metabolic shifts have been observed in a wide variety of microorganisms, including Escherichia coli (Wolfe, 2005), Bacillus subtilis and Corynebacterium glutamicum (Sauer and Eikmanns, 2005), Lactococcus lactis (Thomas et al., 1979; Neves et al., 2005), Saccharomyces cerevisiae (Postma et al., 1989; Huberts et al., 2012), and in tumour cells (Gatenby et al., 2010); for a recent review, see Goel et al. (2012b).

Many studies on these shifts focus on the regulatory mechanisms, such as changes in redox potential (Vemuri et al., 2006), effects on gene expression (Daran-Lapujade et al., 2004) or differential enzyme activity (Thomas et al.,...
1979). However, the evolutionary benefit i.e. the function of switching between these alternative pathways remains puzzling. It has been proposed that the production of acids or alcohol, which often accompanies low-yield metabolism, inhibits growth of competing species (Loesche, 1986; Piskur et al., 2006), but this explanation can be dismissed based on evolutionary arguments (Goel et al., 2012b). We and others have argued that shifting from high- to low-yield metabolism is a consequence of optimal resource allocation that aims to maximise the growth rate — within the boundary conditions imposed by fundamental biophysical and biochemical constraints (Beg et al., 2007; Molenaar et al., 2009; Zhuang et al., 2011; You et al., 2013). Such fundamental constraints could be upper limits to intracellular and/or intra-membrane protein concentrations, or diffusion limitations. Natural selection then results in the highest growth rate per unit of the constrained resource — the allocation of which needs to be optimised, like protein and/or membrane surface.

Various experiments showed that changes in protein synthesis affect fitness, and this has led to the conclusion that the optimisation of the use of protein is an immediate consequence of natural selection (Dean et al., 1986; Dong et al., 1995; Snoep et al., 1995; Dekel and Alon, 2005; Stoebel et al., 2008; Shachrai et al., 2010). Using a self-replicator model that integrates several cellular subsystems as well as the fundamental constraints mentioned earlier, we predicted that a trade-off between protein investment and metabolic yield could explain the choice of metabolic strategy in a growth-optimised microbial system (Molenaar et al., 2009). This and similar models predict that the cell allocates protein to alternative metabolic pathways depending on the extracellular substrate concentration, and consequently, growth rate, thereby altering the metabolic profile (Beg et al., 2007; Zhuang et al., 2011).

If this reasoning were true, we expect a careful tuning of protein investment according to cellular need, as was recently shown for ribosomes in E. coli (Scott et al., 2010). For shifts in metabolic strategies, a change in protein investment between high- and low-yield pathway proteins would be expected as a function of growth rate. Only a few experimental studies have investigated a metabolic shift comprehensively and at multiple cellular levels (Kayser et al., 2005; Castrillo et al., 2007; Haverkorn van Rijsewijk et al., 2011; Huberts et al., 2012). This is what we have done in this work; we chose the lactic acid bacterium L. lactis for two reasons.

First, it exhibits an anaerobic metabolic shift, between lactate production and production of a set of compounds collectively known as ‘mixed acids’ (formate, acetate and ethanol). The strict dependency on substrate-level phosphorylation allows an accurate estimation of ATP production rates. The lactate and mixed acid producing pathways have different ATP yields and likely also different ATP production rates per amount of protein (Molenaar et al., 2009). L. lactis shifts from mixed acid production and high ATP yield to lactate production and lower ATP yield when changing from low to high growth rates in a glucose-limited chemostat (Thomas et al., 1979). Second, the low ATP yields in fermentation ensure that growth of L. lactis is dependent on large fluxes through glycolysis and the alternative fermentation pathways, and hence, one should expect high costs associated with the synthesis of the enzymes involved. This effect is further enhanced because carbon is almost exclusively used for catabolic energy demands as the medium is supplied with all amino acids.

We therefore studied the growth rate-dependent metabolic and protein investment strategy in L. lactis MG1363 using glucose-limited chemostats at different dilution rates. The data consist of high-quality replicate flux, transcriptome, proteome and enzyme activity measurements, all taken from the same chemostats at the same time (Fig. 1). Against our expectations, during a smooth shift at the metabolic level from mixed acids to lactate, and despite a more than threefold change in growth rate, we observed very little regulation at the gene or protein level. These results question the generality of the protein allocation model.

Results

Bioenergetics of the metabolic shift

To study the shift from mixed-acid to homolactic fermentation we cultivated L. lactis (n = 3) under anaerobic conditions in glucose-limited chemostat cultures set at dilution rates (D, henceforth also referred to as growth rate) of 0.15 h⁻¹, 0.3 h⁻¹, 0.5 h⁻¹ or 0.6 h⁻¹. Under these conditions the maximal growth rate was 0.74 h⁻¹ based on wash-out kinetics. At the highest growth rate of 0.6 h⁻¹ the culture primarily yields lactate, whereas at the lowest growth rate of 0.15 h⁻¹ it mainly performs mixed-acid fermentation (Fig. 2A). The fraction of carbon flux directed towards lactate (normalised to the total glucose flux) increased with higher growth rates and ranged between 10% and 75%. At intermediate growth rates (0.3 h⁻¹ and 0.5 h⁻¹), we observed a combination of mixed-acid and lactic acid fermentation. Mixed-acid fermentation yields 3 ATP per glucose, while homolactic fermentation yields only 2 ATP per glucose. Yet, a 65% increase in lactate flux, which corresponds to a 33% decrease in ATP generated per glucose, was not accompanied by a drastic decrease in biomass concentration in the chemostat. It declined only 14% at D = 0.6 h⁻¹ (homolactic fermentation) compared with D = 0.15 h⁻¹ (mixed-acid fermentation; Table 1, Table S5).

The consumption rates of most amino acids increased proportionally with the growth rate (Table S7). Arginine
consumption, however, increased and then dropped with growth rate. At 0.15 h⁻¹, arginine was consumed at a rate of 0.28 mmol·gDW⁻¹ h⁻¹, which gradually increased up to 0.8 mmol gDW⁻¹ h⁻¹ at D = 0.5 h⁻¹ and abruptly dropped at D = 0.6 h⁻¹. Parallel to the consumption of arginine, production of ornithine, citrulline and ammonia steadily increased and then also dropped (Fig. 3B). These data reflect the activity of the arginine deaminase pathway (Fig. 3A), in which arginine is converted via citrulline to ornithine, ammonia and CO₂ with concomitant production of ATP (Driessen et al., 1987; Poolman et al., 1987).

The apparent catabolic and total carbon balances were around 80–90% (Table 1). To obtain a complete view, we calculated the ATP production using a genome-scale sto-
chiometric network model (Verouden et al., 2009; Flahaut et al., 2013) as described by Teusink et al. (2006). This comprehensive method takes contributions from amino acid catabolism into account, and resulted in a steeper increase in ATP production at higher growth rate than when substrate level phosphorylation alone was considered (Fig. S7). These results indicate that amino acid catabolism significantly contributes to the overall bioenergetics in our setting. The total ATP production levelled off and the biomass yield on ATP increased to 17.3 ± 1.4 gDW molATP⁻¹ at D = 0.6 h⁻¹ (Fig. 2B) suggesting that cells grow faster at the same ATP production rate. The reason for this apparent increase in efficiency with respect to ATP is not understood.

Glycolytic fluxes do not correlate with V_max, protein or transcript abundance

We estimated all metabolic fluxes from the genome-scale metabolic model (Flahaut et al., 2013), by averaging the flux ranges predicted by flux variability analysis after constraining all observed nutrient consumption and product formation rates. Flux variability analysis allows calculating upper and lower bounds of inferred fluxes under the constraints imposed by measured fluxes. The estimated glycolytic flux increased proportionally with growth rate, as expected (Fig. 4). The fluxes through lactate dehydrogenase (LDH) increased, while fluxes through pyruvate formate lyase (PFL), acetate kinase (ACK) and alcohol dehydrogenase (ADH) in the mixed-acid-branch increased until D = 0.3 h⁻¹ and then decreased at the higher growth rates.

In contrast to these changes in fluxes, the V_max’s measured by enzymatic assays, as well as the protein and transcript ratios hardly changed (Fig. 4, Tables S1 and S2). Most genes encoding glycolytic enzymes showed hardly any regulation at the transcriptional level, except for a few of which expression increased at the highest growth rate of 0.6 h⁻¹ (Fig. 4). These exceptions were pgIA (phosphoglucone isomerase, PGI), tbaA (aldolase, ALD) and pgmA (phosphoglycerate mutase, PGM) (Table S1). The V_max’s

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Biomass (gDW L⁻¹)</th>
<th>Catabolic C balance %a,b</th>
<th>C balance %a,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.81 ± 0.08</td>
<td>84.0 ± 10</td>
<td>84.53 ± 12.07</td>
</tr>
<tr>
<td>0.3</td>
<td>0.83 ± 0.09</td>
<td>80.0 ± 9.8</td>
<td>84.07 ± 11.82</td>
</tr>
<tr>
<td>0.45d</td>
<td>0.80 ± 0.07</td>
<td>86.0 ± 10</td>
<td>89.46 ± 11.89</td>
</tr>
<tr>
<td>0.5</td>
<td>0.74 ± 0.02</td>
<td>81.7 ± 4.8</td>
<td>86.19 ± 5.28</td>
</tr>
<tr>
<td>0.6</td>
<td>0.70 ± 0.04</td>
<td>80.2 ± 6.7</td>
<td>83.62 ± 8.5</td>
</tr>
</tbody>
</table>

Values represent average of n = 3 except for 0.5 (n = 2) and 0.45 h⁻¹.

a. % C balance = 100 × q_C-out/q_C-in

b. Apparent catabolic C balance calculated from products formed (lactate, pyruvate, ethanol, acetate and succinate) and glucose consumed.

c. Including biomass = 27.8 gDW/C-mole (Oliveira et al., 2005) and amino acids consumed.

d. Single experiment, technical standard deviations reported.
and protein concentration ratios, relative to $D = 0.15 \ h^{-1}$, were also nearly constant for most glycolytic enzymes up to $D = 0.5 \ h^{-1}$. Between $D = 0.5 \ h^{-1}$ and $D = 0.6 \ h^{-1}$ the $V_{max}$'s and protein ratios of glycolytic enzymes encoded by the las operon (phosphofructokinase (PFK), pyruvate kinase (PYK) and LDH), and of fructose bisphosphate ALD and triosephosphate isomerase (TPI) increased. $V_{max}$'s of PGI, phosphoglycerate kinase (PGK) and PGM also increased, but the changes in $V_{max}$ were small compared with the changes in glycolytic flux.

The enzymes of the mixed-acid fermentation pathway did show some gene and protein regulation, with the expected decreasing trend with increasing growth rates. PFL protein ratios decreased linearly above $0.15 \ h^{-1}$. Protein concentration ratios of phosphotransacetylase (PTA) and $V_{max}$'s and concentration ratios of ACK and ADH
Fig. 4. Schematic overview of the glycolytic pathway and downstream metabolic conversions. The transcript and protein ratios, the $V_{\text{max}}$'s of the corresponding enzymes, the metabolic fluxes and the $V_{\text{max}}$/flux ratios per enzyme are plotted as functions of growth rate; the data are normalised to $D = 0.15 \text{ h}^{-1}$. Grey areas represent standard errors. For fluxes with isoenzymes, those with the highest protein abundance are shown: GAPDH, GapB; PGM, GpmA; ADH, AdhE; ACK: AckA1(▲) and AckA2(●).
dropped at $D = 0.6 \text{ h}^{-1}$ compared with the other D's. The two copies of ACK displayed opposite regulatory trends. At higher growth rate, the ackA1 transcript increased while the AckA1 protein ratio stayed constant, whereas the ackA2 transcript remained constant, while the relative AckA2 protein ratio decreased.

The correlation between the transcriptome and the proteome for all pooled chemostat data was generally low, with $r = 0.262$. Although this correlation is higher for some functional categories (Fig. S9 and Table S4), overall the changes in protein and transcript ratios were not proportional. Low correlations between mRNA and protein ratios have been observed more often (Picard et al., 2009; Taniguchi et al., 2010). The correlation between enzyme activities and their respective protein ratios as measured in the proteome studies are shown in Fig. 5A. Enzymes showed good correspondence between protein ratios and enzyme activities, with GLK, ACK and ADH as notable exceptions. Clearly these changes did not generally correlate with the changes in fluxes.

**Metabolic shift is predominantly regulated at the enzyme activity level**

Apparently, the flux in glycolysis and in the acid production branches is hardly regulated by protein concentrations. This conclusion can be quantified by the hierarchical and metabolic regulation coefficients (ter Kuile and Westerhoff, 2001). The hierarchical regulation coefficient ($\rho_m$) is defined as the relative change in flux over the relative change in $V_{\text{max}}$. It will be equal to 1 if $V_{\text{max}}$ and flux would change proportionally. The metabolic regulation coefficient ($\rho_h$) is defined as the complement $\rho_h = 1 - \rho_m$ (see **Experimental procedures**). Except for the comparison of $D = 0.5 \text{ h}^{-1}$ and $D = 0.6 \text{ h}^{-1}$, where the $V_{\text{max}}$ value increases, the $\rho_m$'s were close to 1 (Fig. 5B) because the flux increased substantially while the $V_{\text{max}}$ remained almost unchanged. From $D = 0.15 \text{ h}^{-1}$ to $D = 0.5 \text{ h}^{-1}$ all the $\rho_m$'s were above 0.8. From $D = 0.3 \text{ h}^{-1}$ to $D = 0.6 \text{ h}^{-1}$, the $\rho_m$'s were lower, indicating partial metabolic and partial hierarchical regulation.

From $D = 0.5 \text{ h}^{-1}$ to $D = 0.6 \text{ h}^{-1}$, except for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PTA and ACK, all the $\rho_m$'s are zero, indicating similar fold-changes in fluxes and $V_{\text{max}}$'s at these growth rates (hierarchical regulation). The $\rho_m$'s of ADH for all growth rate comparisons except $0.15$–$0.5 \text{ h}^{-1}$ are below zero, indicating a high degree of hierarchical regulation of this enzyme over the entire range of growth rates measured here.

If protein cost would have been a main driver in the evolution of the regulation of glycolysis, the expected outcome of this experiment would have been that in vitro $V_{\text{max}}$'s and fluxes are highly positively correlated, and consequently, that the hierarchical regulation coefficients are high over a wide range of growth rates. This is clearly not the case.
Ribosome investment

Models of bacterial growth predict a proportional increase of the proteins allocated to ribosomes (rProtein) with the growth rate (Maaløe and Kjeldgaard, 1966; Gausing, 1977; Molenaar et al., 2009). Ribosome abundance is quantified by the ratio of totRNA over the total amount of protein (totProt), as ribosomal RNA (rRNA) represents approximately 85% of the total RNA (totRNA) (Bremer and Dennis, 1996; Scott et al., 2010). We determined the ratio totRNA/totProt as an estimate of the ribosomal content of L. lactis cells and observed that this ratio increased, but not in proportion to the growth rate. The increase in ribosomal content relative to that at the lowest growth rate levels off at the highest growth rate of \( D = 0.6 \, h^{-1} \) (Fig. 6A). The protein and transcript ratios of the rProteins and their respective genes increase only slightly when compared with those at the lowest growth rate, with the protein ratios increasing somewhat steeper than the corresponding mRNA ratios (Fig. 6B and C).

Discussion

Motivation for the experimental setup

We characterised the growth rate-dependent regulation of metabolic strategies of the model lactic acid bacterium L. lactis MG1363 in unprecedented detail and at multiple molecular levels. Like others (Thomas et al., 1979), we observed a shift from mixed-acid fermentation at low growth rates to homolactic fermentation at high growth rates, which represents an anaerobic equivalent to the (bacterial) Crabtree or Warburg effect (Warburg, 1956; DeBerardinis and Thompson, 2012). Only a few quantitative studies are available that link growth rate of L. lactis to such metabolic responses. One of these investigated amino acid metabolism in isoleucine-limited chemostat cultures of L. lactis IL1403 (Dressaire et al., 2008) while another examined glucose limitation in L. lactis IL1403 in an accelerostat setup (Lahtvee et al., 2011). Neither observed a metabolic shift to mixed-acid fermentation at the lower growth rates, probably because of the nature of the strain used in these studies, e.g. different PFL and ADH enzymes (Even et al., 2001).

We designed our experiments specifically to test the hypothesis that shifts in metabolic strategies are outcomes of evolutionary optimisation of resource allocation, in particular to resources for protein synthesis or occupation of volume by protein (Beg et al., 2007; Molenaar et al., 2009). The investment in protein associated with glycolysis is considerable. Using ratios of total mass spectra signals in the proteome data of the soluble protein fractions as an indication of fractions of total protein, we calculated that 18–23% of total soluble proteins are glycolytic proteins (Fig. 7). This is in accordance with an earlier observation (Guillot et al., 2003). Another major protein fraction represents ribosomal proteins encompassing 19–28% of the total soluble protein pool. The total protein investment in glycolytic and ribosomal proteins therefore varies between 40% and 48%. We reasoned therefore that, if optimal allocation of resources for protein synthesis to the different protein fractions was an important determinant in maximisation of fitness, as has been proposed and shown by others (Dean et al., 1986; Dekel and Alon, 2005), one would expect significant regulation of glycolytic and ribosomal proteins in L. lactis over the range of growth rates investigated. Specifically, proportionality between growth rate and ribosomal protein and between glycolytic enzymes and glycolytic flux would be expected (Molenaar et al., 2009).

Fig. 6. Lactococcus lactis ribosome investment as a function of growth rate.
A. The log₂ change of totRNA/totProt ratio relative to that at the lowest growth rate 0.15 h⁻¹. Grey areas represent standard errors.
B. The log₂ mRNA ratios of rProteins.
C. The log₂ rProtein ratios from the combined soluble and membrane proteome, relative to \( D = 0.15 \, h^{-1} \).
Physiological characterisation of the experimental conditions

While we indeed observed a smooth transition at the metabolic level between mixed-acid and lactate fermentation, we observed relatively small changes at the transcript and proteome levels with only more abrupt (but still rather mild) changes between \( D = 0.5 \) h\(^{-1} \) and \( D = 0.6 \) h\(^{-1} \). The latter changes likely result from glucose-induced regulation, notably catabolite repression (Fig. S6). At \( D = 0.6 \) h\(^{-1} \) \textit{L. lactis} approaches its limit of growth, which is \( 0.74 \) h\(^{-1} \) for the medium used here (Fig. S5). As glucose was the growth-limiting nutrient in our chemostat setting (Fig. S3), the concentration of residual glucose in the chemostats changed quite drastically from undetectable in the chemostats at \( D = 0.15 \) h\(^{-1} \) up to \( D = 0.5 \) h\(^{-1} \), to a few mM at \( D = 0.6 \) h\(^{-1} \) (Table S5).

The effect of glucose-induced catabolite repression is probably best illustrated by the regulation of arginine catabolism, where steadily increasing levels of transcripts and proteins of the arginine catabolic pathway plummeted at \( D = 0.6 \) h\(^{-1} \), as did the consumption rates of arginine and the production rates of ornithine, citrulline and ammonia (Fig. 3B). The upstream region of the arginine catabolic gene cluster \textit{arcABD1C1C2TD2} contains 6 ARG boxes for binding of ArgR (Larsen et al., 2008), a putative CodY operator site and a catabolite repression cre-site for CcpA binding (Zomer et al., 2007). It was proposed that arginine binds to the arginine regulator AhrC to promote derepression of the \textit{arc} cluster (Larsen et al., 2004). We observed that until \( D = 0.5 \) h\(^{-1} \), residual arginine levels are a monotonic increasing function of the growth rate (Fig. 3C): the enhanced expression of ArcA fits with such a scenario. At the highest growth rate, however, CcpA-mediated catabolite repression must override this regulation. An alternative explanation, namely that arginine demand at a high growth rate is so high that intracellular levels drop, is rather unlikely as no significant upregulation was recorded of the arginine biosynthetic genes \textit{argCJBF, argGH} and \textit{gltSargE}.

Thus, with our setup, we seem to have created over a continuous range of fluxes and growth rates two nutritional regimes that correspond to a low glucose concentration regime (famine) and a high-glucose concentration regime (feast). In view of the differences in fluxes, the observed expression differences are remarkably low. Recently it was suggested for \textit{E. coli} that intracellular fluxes are sensed by flux sensors that regulate gene expression accordingly (Kotte et al., 2010). Our results suggest that whichever intracellular flux sensors may be operative in \textit{L. lactis}, they do not seem to be active in the flux regime explored here. Rather, for many genes, the binary (low-high) extracellular glucose levels may explain their expression patterns.

Growth rate and ribosomal protein regulation

When the protein content of biomass is relatively constant, the total protein synthesis rate should increase proportionally to the growth rate. If under the same conditions the quantity of ribosomes increases less than proportionally, i.e. the relation between the amount of ribosomes and growth is concave as in this study (Fig. 6A), then the peptide elongation rate and/or efficiency of protein synthesis should increase with the growth rate. Note, however, that proportionality does not necessarily imply constant peptide elongation efficiency. A similar concave relation was found in \textit{E. coli}, but only at low growth rates: the relation between the amount of ribosomes and growth rate was proportional over a large range of growth rates from \( 0.6 \) h\(^{-1} \) to \( 2.2 \) h\(^{-1} \) (Gausing, 1977).

The idea of a variable overall elongation efficiency of the ribosomes is consistent with the finding that ribosomal proteins showed differential albeit low expression. Moreover, ribosomes can hibernate at low growth rates by forming dimers through contacts between their 30S subunits. This phenomenon has been observed in \textit{E. coli}, \textit{Staphylococcus aureus} and \textit{B. subtilis} (Ueta et al., 2005; 2010; Tagami et al., 2012), and in mammalian cells (Krokowski et al., 2011). In \textit{L. lactis}, the YfiA protein is necessary and sufficient for ribosome dimerisation (Puri et al., 2013), and we indeed observed higher \textit{yfiA} transcript levels at low growth rates.

We might have taken the wrong measure for ribosome abundance. Most RNA in a bacterial cell is rRNA and hence the \textit{totRNA/totProtein} ratio is considered a good indicator of the amount of ribosomes. As many ribosomal

\[
\text{Fraction of total signal}
\]

\[
\text{Gene class}
\]

- glycolysis
- ribosome
- elongationfactor

\[
\text{Dilution rate (h}\^{-1}\text{)}
\]

Fig. 7. Estimated fractions of cytosolic protein involved in glycolysis and protein biosynthesis. The estimates are based on the sum of mass spectra signals in the proteome of all peptides originating from these classes of proteins relative to the total sum of all peptide signals in the soluble fraction.
proteins can be removed without loss in ribosome activity (Noller et al., 1992; Steitz and Moore, 2003; Klein et al., 2004; Bubunenko et al., 2006), the quantity of total ribosomal protein may not be a good indicator for the total amount of functional ribosomes. The so-called early-assembly rProteins are thought to structure rRNA such that the complex functions as a ribosome (Nierhaus, 1991). The amount of early-assembly rProteins shows a gradual increase with increasing growth rate (Fig. S1), but this increase is not proportional to the rRNA abundance (Fig. 6A). Similar trends are observed in the relative ratios of rProteins in E. coli growing at different growth rates (Valgepea et al., 2010), but it is premature to conclude that a lower rProtein requirement at high growth rates is a general phenomenon in bacteria.

**Growth rate, glycolytic flux and glycolytic enzymes**

The glycolytic flux increased proportionally with the growth rate. This was consistently not the case for the levels of the corresponding transcripts and proteins, and for enzyme activities. The ratios of $V_{\text{max}}$/flux showed that the enzyme capacities were generally much higher than the actual flux in the cell at all growth rates and for all measured enzymes except for PGM, GAPDH and enolase (Fig. 4). The in vitro assays for the latter three enzymes presented technical issues, as detailed in the Experimental procedures section. For the other enzymes, the overcapacity could accommodate a fourfold increase in the flux. Such a lack of correspondence between flux and gene expression in central metabolism was also observed in *B. subtilis* during growth on various carbon sources (Chubukov et al., 2013) and in yeast growing at different glucose concentrations (Hoek et al., 1998). Presumably, an increase in residual glucose is sufficient to increase the flux through the glycolytic enzymes, as was observed also for yeast growing at different temperatures (Tai et al., 2007).

What is perhaps more surprising is that the enzymes of the mixed-acid branch and the lactic acid branch are also present in excess, even though we observed a switch in usage between these pathways. We do see trends in expression in the right directions, but these seem to be rather weak in relation to the flux changes. This observation is in direct contrast to the argument that spending protein on a pathway that is hardly used would be detrimental to fitness. Exceptions are ADH and PFL. ADH seems to be truly regulated at the expression (transcription and translation) level. PFL activity could not be measured as the enzyme is prone to oxidative inactivation (Melchiorssen et al., 2000), but it was shown before that the PFL protein level correlates with the flux through the mixed-acid pathway (Melchiorssen et al., 2002). Indeed, the protein ratios of PFL decreased with increasing growth rate and decreasing flux through the mixed-acid pathway (Fig. 3), suggesting hierarchical regulation.

For ACK, we observed opposite trends for the two iso-enzymes AckA1 and AckA2. AckA1 is the preferred enzyme for ATP formation, while AckA2 is primarily involved in acetate assimilation and synthesis of acetyl-phosphate (Chan et al., 2014; Puri et al., 2014). These different roles may explain the differential regulation of the ackA transcripts.

From our data, it appears that *L. lactis* does not change its metabolic strategy because of protein synthesis economy. Rather, it keeps glycolytic activity and both metabolic branches highly expressed and uses either post-translational modifications or metabolic effectors to change flux. A recent study revealed phosphorylation of *L. lactis* proteins at amino acid residues serine, threonine and tyrosine (Soufi et al., 2008). In the absence of post-translational modification we should find a perfect correlation between $V_{\text{max}}$’s and protein levels, as $V_{\text{max}}$ is a product of total enzyme concentration and the catalytic turnover number ($k_{\text{cat}}$). We do find such correlations, with a few exceptions (Fig. 5A). For the other enzymes, metabolic regulation appears the way to change flux.

**Overcapacity of proteins and ribosomes: explanations**

To conclude, there seems to be an excess of glycolytic enzymes and ribosomes at low growth rates, which, from a point of view of optimising protein allocation to maximise growth rate, seems inefficient. The view that is emerging for *E. coli* (Scott and Hwa, 2011; Klumpp and Hwa, 2014), that regulation strategies can be viewed as resource allocation problems to allow maximal specific growth rate, appears too simple for *L. lactis*. Rather, the (lack of) extensive regulation of the levels of glycolytic and ribosomal proteins shows that protein costs hardly play a role in determining fitness of this organism under these conditions. What are the possible explanations for the observed expression patterns?

The apparent overcapacity of ribosomes at low growth rates in *E. coli* was suggested to be an ‘optimistic strategy’, allowing a faster growth response when conditions improve thereby increasing the overall growth rate in a fluctuating environment (Gausing, 1977). For yeast, it has also been suggested that it might be beneficial under dynamic conditions to switch between alternative pathways rather than to continuously break down enzymes of the least optimal pathway (Tai et al., 2007). Thus, *L. lactis* and other microorganisms may not be optimally geared towards the constant conditions prevailing in a chemostat, but rather, have evolved strategies for a dynamic environment.

Indeed, an inherent assumption of the self-replicator model is that organisms are evolutionarily optimised for a
specific niche with nutrient concentrations varying over a wide range, but not over time. One may wonder whether the low glucose concentrations in the chemostat employed here reflect any condition that \textit{L. lactis} may have met in its evolutionary history. Native to a rich environment of milk, this industrial microorganism has been selected by humans for fast growth (fast acidification) at high sugar (lactose) concentrations, and may thus have lost or never acquired regulation of protein allocation at low substrate concentrations. However, a metabolic flux change in the absence of alterations in protein allocation was recently also detected in other organisms, notably \textit{B. subtilis} (Chubukov et al., 2013). This observation argues against a specific maladaptive behaviour of \textit{L. lactis}.

Perhaps in a glucose-limited chemostat, \textit{L. lactis} may only be able to detect low and high glucose levels, reflecting a bipolar lifestyle of feasting or fasting. Under the latter condition, it just preserves a large overcapacity of ribosomes and glycolytic enzymes to be ready to rapidly respond and grow when conditions improve. Whatever the explanation will turn out to be, it makes the evolutionary origin of a growth rate-dependent and anaerobic bacterial version of the Warburg/Crabtree effect all the more intriguing.

### Experimental procedures

#### Strain and growth medium

\textit{Lactococcus lactis} ssp. cremoris MG1363 (Gasson, 1983) was grown on chemically defined medium for prolonged cultivation (CDMPG) with 25 mM glucose as the limiting nutrient as detailed before (Goel et al., 2012a).

#### Culture conditions

Glucose-limited chemostat cultures were grown in 2 L bioreactors with a working volume of 1.2 L at 30°C, under continuous stirring. The headspace was flushed at 5 headspace volume changes per hour, with a gas mixture of 95% N\textsubscript{2} (99.998% pure) and 5% CO\textsubscript{2} (99.7% pure) with oxygen impurity less than 34 vpm. A pH of 6.50 ± 0.05 was maintained by automatic titration with 5 M NaOH. Fermenters were inoculated with 4% (v/v) of standardised pre-cultures consisting of 45 ml of CDMPG inoculated with 300 μl of a glycerol stock of \textit{L. lactis} MG1363 and incubated for 16 h at 30°C. After batch growth until an optical density at 600 nm (OD\textsubscript{600}) of around 1.8, medium was pumped at the appropriate dilution rate (0.15 h\textsuperscript{-1}, 0.3 h\textsuperscript{-1}, 0.45 h\textsuperscript{-1}, 0.5 h\textsuperscript{-1}, 0.6 h\textsuperscript{-1}). The actual dilution rate was measured shortly before harvesting. Some replicate chemostat cultures had a slightly different dilution rate. The largest deviation was found for a chemostat, which ran at a D = 0.45 h\textsuperscript{-1} while being set at a D = 0.5 h\textsuperscript{-1} (Table 1). However, as the transcriptome sample derived from this chemostat did not differ significantly from the two other replicates, the three samples were, for the benefit of statistical power, treated as biological replicates in the analysis of transcriptome and proteome data.

### Harvesting of cells from chemostats

The chemostats were harvested assuming a steady state at 10 working volume changes (Even et al., 2003). At harvest, the medium inflow was stopped and the entire culture in the chemostat was pumped out at a high flow rate into sampling tubes placed on ice; the whole procedure taking less than 90 s. Samples were collected for cell density, extracellular metabolite analysis, DNA microarray analysis, enzyme activity assays, and finally for proteomic and fatty acid composition analysis.

#### Cell density

Cell density was measured spectrophotometrically at 600 nm and calibrated against cell dry weight measurements (n = 3) performed for each sample as follows. Four millilitres of culture was filtered through a pre-dried, pre-weighed 0.2 μm cellulose nitrate filter (Whatman GmbH, Dassel, Germany), washed twice with deionised water and dried to a constant weight. For one unit change of optical density, the change in dry weight was determined to be 0.31 ± 0.02 g L\textsuperscript{-1} OD\textsubscript{600}\textsuperscript{-1}.

#### Fermentation end-product, ammonia and amino acid analysis

Supernatant samples from medium bottles and chemostat cultures were prepared by filtering through a 0.2 μm poly-ethersulfone filter (VWR international B.V., Amsterdam, The Netherlands) and storing the flow-through at -20°C until further analysis. Extracellular concentrations of lactate, acetate, ethanol, formate and glucose were determined by high-performance liquid chromatography as described in Goel et al. (2012a). Residual glucose concentrations were determined by enzymatic coupling with Nicotinamide adenine dinucleotide phosphate (NADP\textsuperscript{+}) in an assay containing 100 mM HEPES-KOH, 5 mM MgSO\textsubscript{4}, 2 mM ATP, 4.5 mM NADP\textsuperscript{+}, 1.5 U ml\textsuperscript{-1} hexokinase, 1 U ml\textsuperscript{-1} glucose-6-phosphate dehydrogenase (G6PDH) and sample or standard. Ammonia concentrations were measured using a commercially available ammonia assay kit (catalogue no. AA0100, Sigma-Aldrich, St. Louis, MO, USA). Amino acids were analysed by liquid chromatography with fluorescence detection (350 nm excitation, 450 nm emission, RF 20-A, Shimadzu, ‘s-Hertogenbosch, The Netherlands). Precolumn derivatisation using ortho-phthalaldehyde (OPA) and 3-mercaptopropionic acid was applied. The analysis was carried out by online detection using a programmable SIL 10A autosampler (Shimadzu). In brief, 25 μg OPA were dissolved in 5 ml borate buffer (0.1 M, pH 10.2) containing 21 μl 3-mercaptopropionic acid. Twenty-five microlitres of sample was mixed with 25 μl of borate buffer (0.2 M, pH 10.2) and 25 μl of a DL-norvaline internal standard solution (1 mM) was added. The sample is made up to 950 μl with MilliQ (Millipore, Amsterdam, The Netherlands) water and placed in the autosampler. To every sample the SIL 10A autosampler adds 50 μl of OPA solution before analysis. Analysis was carried out using a quaternary LC 20AB pump (Shimadzu) operated at 640 μl min\textsuperscript{-1}. The pump delivered a gradient of 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}, pH 8.2 in MilliQ water, containing 5 mM NaN\textsubscript{3} (eluents A) and
Cells (2 × 30 ml) were harvested by centrifugation (5 min, 4500 g); pellets were immediately frozen in liquid nitrogen and stored at −80°C. For RNA isolation the frozen cells were thawed on ice. Subsequent cell disruption, RNA purification, reverse transcription and Cy3/Cy5 labelling were done as described previously (Larsen et al., 2006). Labelled cDNAs were hybridised to full-genome DNA microarray slides of L. lactis MG1363 (Kuipers et al., 2002), with the addition of probes for rProteins. All reagents and glassware for RNA work were treated with diethyl phosphorocyanidate (DEPC). DNA, cDNA quantity and quality, and the incorporation of the cyanine-labels were examined by NanoDrop (Thermo Fisher Scientific, Inc., Rockford, IL, USA) at 260 nm for RNA and cDNA, 550 nm for Cy3, and 650 nm for Cy5. The four chemostats with increasing growth rate were run as biological triplicates. Thus, three times the samples of an increasing growth rate were compared directly with each other in combination with a dye-swap (Fig. 6, hybridisation scheme). DNA microarray slide images were analysed using ArrayPro 4.5 (Media Cybernetics, Inc., Silver Spring, MD, USA). Filtering of bad- and low-intensity spots and signals, data parsing, automated grid-based Lowess normalisation, scaling, data visualisation and outlier detection were performed using the Limma-package (Smyth, 2005). We used the common reference design in which direct and indirect comparisons were used to increase statistical significance. Fold-changes are considered to be significantly altered when the P-value ≤ 0.05.

Enzyme activities: sampling, cell extract preparation and assay conditions

A volume of cell culture containing 100 mg dry weight was centrifuged (4°C, 5 min, 8000 r.p.m.), washed once and resuspended in 3–6 ml 50 mM HEPES-KOH at pH 7.5, containing 15% glycerol supplemented with Halt Protease Inhibitor single-use cocktail, Ethylenediaminetetraacetic acid (EDTA)-free (Thermo Fisher Scientific, Inc.). This suspension was divided into 0.5 ml aliquots added to 0.5 mg glass beads with 100 μm diameter (BioSpec Products, Bartlesville, OK, USA) in screw-capped tubes, snap-frozen in liquid nitrogen and stored at −20°C until further analysis. Frozen samples were thawed on ice and MgCl₂ was added to a final concentration of 2 mM. Cells were disrupted in a FastPrep FP120 homogeniser (BIO 101, Vista, CA, USA) at a speed setting of 6, in three bursts of 20 s, with 120 s intermittent cooling. After centrifugation (4°C, 10 min, 10,000 g), the supernatant was collected and a series of dilutions were prepared, which were used immediately for enzyme assays. Protein concentrations of cell extracts were determined on the same day by the bicinchoninic acid (BCA) method (Stoscheck, 1990) with a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc.) using BSA (2 mg ml⁻¹ stock solution; Pierce), containing 2 mM MgCl₂ and Halt Protease Inhibitor cocktail, as the standard. Enzyme activities were assayed at 30°C at pH 7.5 in freshly prepared cell extracts within 2 weeks of harvesting the chemostats. The enzymes GLK, G6PDH, PGI, PFK, ALD, TPI, GAPDH, PGK, PGM, ENO, PYK, LDH, ACK, PTA, ADH and aldehyde dehydrogenase (ALDH) were assayed with the in vivo-like assay medium (version 1) as described by Goel et al. (2012a) with the following differences: the coupling enzymes were not desalted, GAPDH was assayed with 5 mM arsename (Garrigues et al., 1997) and PGM was assayed in the absence of activator,2,3-bisphosphoglycerate. These differences arose because the chemostat samples were assayed within a week of harvesting to avoid enzyme deterioration, and we had to work with an interim version of the in vivo-like assays was further developed later on. ALDH activity was not detected. All assays were checked for linearity and proportionality with increasing cell extract, with at least n = 4. The values obtained from the assays yield the total activity of all isoenzymes in the cell extract and are expressed as the rate of substrate converted, relative to total protein in the extract. Obtained activities in μmol min⁻¹ mg protein⁻¹ converted to fluxes (in mmol gDW⁻¹ h⁻¹) by multiplying activities with the ratio of total protein content per dry weight estimated for each chemostat culture.

Proteomic analysis

For protein expression profiling 2 × 250 ml of culture from each chemostat was collected by directly pouring in it pre-chilled centrifuge bottles containing chloramphenicol at a final concentration of 10 μg ml⁻¹ (2.5 ml of stock solution, 10 mg ml⁻¹). The cells were harvested by centrifugation (4°C, 5 min, 8000 r.p.m.). Supernatant was discarded and the pellet was washed with 50 ml of wash buffer (50 mM HEPES-NaOH pH 7.5, 15% glycerol) and centrifuged. The washed cell pellets were resuspended in 10 ml wash buffer, frozen in liquid nitrogen and stored at −80°C. Cells corresponding to OD600 of 50 in a total volume of 6 ml with 1 mM MgCl₂ were disrupted at 39 Kpsi with a Constant Systems cell disrupter (Constant System Ltd). The crude cell lysates were centrifuged (4°C, 15 min, 12,000 g); the supernatant was carefully recovered and subsequently centrifuged (4°C, 15 min, 267,000 g). The supernatant, containing the soluble fraction was removed and stored at −80°C. The residual membrane fraction was washed once and finally resuspended in 500 μl of wash buffer and stored at −80°C. Protein concentrations for both soluble and membrane fractions were determined with BCA kit (Pierce). For Trypsin digestion 50 μg of protein was resuspended in 50 μl of 500 mM Triethylammonium bicarbonate (TEAB), 2% acetonitrile and 0.08% SDS. The disulfide bonds were reduced with 3 mM Tris (2-
carboxymethyl) phosphine hydrochloride, and the cysteine residues were modified with 4 mM iodoacetamide. The 8-plex iTRAQ labelling was performed three times (Fig. 6), according to the manufacturer’s protocol with few modifications as described in Steen et al. (2010). The peptide mixture was subjected to chromatography and spectrometric analysis. The pre-fractionation of peptides was performed on a silica-based polysulphoethyl aspartamide strong cation exchange column (catalogue number: 202SE0502 Poly LC, Inc., Columbia, MD, USA).

Proteomic data analysis and statistics

Raw proteome for each sample data consisted of four sets of 8-plex iTRAQ signal strengths annotated with a peptide and protein identifiers. Three datasets each originated from membrane and soluble protein fractions. Membrane and soluble protein fraction were analysed separately. Peptide identifiers could only be compared within and not between an 8-plex iTRAQ data set. Individual samples within an 8-plex dataset were signal normalised by LOESS regression on an M-A transformation of the signals, as is common in microarray analysis. The assumption that the bulk of log-transformed signal ratios between different samples or between replicates will ideally be located symmetrically around 0 (no regulation) independent of the signal strength underlies this normalisation technique. As this technique is used originally when comparing only two samples, an adaptation for eight samples was made. LOESS normalisation was performed for each of the 28 unique pairs of samples within an 8-plex set, and these normalisations were reconciled by linear modelling. The normalised data were used to fit the logarithmically transformed ratios of protein amounts at the different growth rates (relative to growth rate 0.15 h⁻¹) taking into account the additional effects of peptide and iTRAQ 8-plex set.

Total cell protein and total RNA

Frozen cells from the proteomic sample of each chemostat were thawed and 100 μl cells were diluted to a constant OD of 0.25. These were further diluted up to a volume of 400 μl and used to estimate total protein and RNA. For total protein quantification, the cells were lysed with 2% SDS and incubated at 96°C for 2 h. Protein concentrations in the obtained cell lysates were determined (n = 3) using a BCA Protein Assay Kit (Pierce, Thermo Fischer Scientific, Inc.) using BSA (2 mg ml⁻¹ stock solution; Pierce) as the standard (Stoscheck, 1990). For total RNA, cells were disrupted with a Qiagen Tissue Lyser (15 Hz, 2 cycles, 5 min each), and total RNA was extracted with phenol/ chloroform/isoamylalcohol (25:24:1 v/v), and extracted again with chloroform/ isoamylalcohol (24:1 v/v). The total RNA was precipitated by the addition of isopropanol and by adding potassium acetate to a final concentration of 150 mM, supplemented with DEPC. Following by vacuum-centrifugation for removal of the solvents from the RNA. Finally, samples were completely dissolved in MilliQ-DEPC and total RNA was quantified by measuring absorbance 260 nm using NanoDrop (Thermo Fisher Scientific, Inc.). Data reported is an average of technical duplicates for each biological sample.

Regulation analysis

We used regulation analysis (ter Kuile and Westerhoff, 2001) to investigate the growth rate-related flux regulation in L. lactis. The hierarchical regulation coefficient (ρₘ) represents the extent of flux regulation through gene expression and via changes in enzyme concentration. It can be defined as,

\[ \rho_m = \frac{\Delta \ln J}{\Delta \ln x} = \frac{\Delta \ln e}{\Delta \ln J} \]

for a pathway flux J, with concentration x of enzyme i, which carries a flux at a rate V. The extent of flux regulation of enzyme activity by metabolite x is called the metabolic regulation coefficient (ρₜ) defined as,

\[ \rho_t = \frac{\Delta \ln V}{\Delta \ln x} = \frac{\Delta \ln e}{\Delta \ln J} \]

At steadystate, the sum of the regulation coefficients ρₘ and ρₜ is 1.

The metabolic regulation coefficient ρₜ was calculated via ρₘ, which was computed for a set of two growth rates, as the ratio of the difference between the logarithms of the fluxes to the difference between the logarithms of the enzyme activities, at both growth rates.

Constraint-based modelling: flux balance analysis and flux variability analysis

The genome-scale stoichiometric network model was based on an existing model of L. lactis MG1363 (Verouden et al., 2009; Flahaut et al., 2013) with modifications in growth and maintenance energy parameters, which were estimated as described earlier (Teusink et al., 2006). The network was constrained with all measured experimental fluxes with the objective of maximising ATP dissipation to estimate the maintenance coefficient as the maximum ATP dissipation rate, and the ATP requirement for precursor biosynthesis was estimated by the reduced cost of biomass flux for ATP dissipation. This exercise was repeated to calculate the ATP parameters for each chemostat culture at each growth rate resulting in 12 models (for general models, and upper and lower bounds for each simulation, see Supporting Information, and Table S9). Flux variability analysis at a fixed growth rate was carried out for all models and the flux distribution was obtained by calculating the average of the flux range for each individual flux. All analyses were carried out using the web-based modelling tool: Flux Analysis and Modelling Environment (Boele et al., 2012). The model descriptions have been added in the supplementary material according to the SBML level 2 standard.

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**Author contributions**

AG carried out fermentations, enzyme activity measurements and constraint-based modelling with help from BT, analysed the extracellular metabolome with help from MG and FS, and wrote and drafted the paper. TE performed microarray analyses with help from AdJ, OPK and DM in data analysis, total RNA extraction, and fatty acid analysis, and wrote the paper. PP carried out the total protein extraction and proteomics analyses, with help from FF in measurements, and DM in data analysis. DM performed the multi-level data analysis. BT, DM and OPK conceived the study, and FS, JK, DM, WdV, BP, OPK and BT co-designed the experimental approach and critically revised the paper. All authors read and approved the final paper.

**Conflict of interest**

None declared.

**References**


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