Chapter 3

Metabolic regulation governs the metabolic shift from mixed-acid to homolactic fermentation of *Lactococcus lactis*: A multi-level omics study

Thomas H. Eckhardt, Anisha Goel, Pranav Puri, Filipe Santos, Douwe Molenaar, Willem M. de Vos, Jan Kok, Bert Poolman, Anne de Jong, Fabrizia Fusetti, Bas Teusink and Oscar P. Kuipers
Abstract

Using a simple model bacterial system, we test the hypothesis that investment in protein synthesis influences the metabolic strategy employed. We characterized the physiological responses of the bacterium Lactococcus lactis growing in glucose-limited chemostats at various growth rates as relative transcription and protein ratios, enzyme activities and fluxes. A drastic shift in carbon flux from 10 to 75% lactate formation was accompanied by very limited changes in transcription, protein ratios or even enzyme activities, in glycolytic and fermentative pathways. These minimal changes were reflected even in the ribosomal proteins, a major investment of cellular machinery. Thus, contrary to the original hypothesis, L. lactis displays a strategy in which its central metabolism appears always prepared for high growth rate and in which it primarily employs regulation of enzyme activity rather than alteration of gene expression. Only at the highest growth rate and during batch growth we observed down-regulated stress proteins and up-regulated glycolytic proteins - triggered by glucose excess. We conclude that for glucose, transcription and protein expression largely follow a binary feast / famine logic.

Introduction

Microorganisms synthesize various metabolites depending on their growth conditions. Under certain conditions, they also undergo metabolic shifts. The occurrence of metabolic shifts in a wide variety of (micro)organisms has been investigated extensively; for a recent review see. Among the multitude of theories and explanations about metabolic shifts, several consider one or more aspects of microbial physiology important, e.g. metabolism, gene expression, or competitive advantage, to name only a few. Others suggest the importance of biochemical constraints, spatial structure and limited intracellular and membrane space. In light of the many studies emphasizing the role of protein cost in overall cellular behaviour, one cannot help to wonder about a possible relationship with the metabolic shift. The theories of limited intracellular and membrane space translate to protein investment influencing the metabolic shift. Similarly, protein investment and metabolism were linked and the suggestion is that evolutionary optimization of resource allocation underlies the
metabolic shift. So, a self-replicating system integrating several cellular subsystems was proposed. The predictions of this self-replicator model lead to the hypothesis that a trade-off between protein investment and metabolic yield ultimately governs the metabolic strategy in a growth-optimized microbial system. Depending on the proteins involved in the different metabolic pathway branches, investment of proteins (enzymes) varies with varying substrate availability and consequently growth rate, altering the metabolic profile of the microorganism. To test this hypothesis, a good model system exhibiting a metabolic shift and the ability to quantify protein investment would be necessary. Surprisingly, only few experimental studies investigate the metabolic shift at multiple cellular levels. We chose the model lactic acid bacterium Lactococcus lactis because of its classic mixed-acid-to-lactic-acid metabolic shift, its simple metabolism without respiration (in the absence of exogenously supplied hemin), its relatively small and well-characterized genome and its industrial importance. It exhibits the shift between mixed-acid and homolactic fermentation upon changing growth rate under the same steady state environmental conditions. This is inherently different from the other well-studied microbial model organisms, yeast, E. coli and B. subtilis, with respect to the fact that L. lactis does not show a diauxic shift upon using the overflow metabolites. L. lactis also lacks an electron transport chain and the capacity to respire under normal conditions, and hence the ATP gain in the metabolic shift from mixed acids to lactic acid is over 10 times smaller. A well designed multi-level-omics study can provide sufficient data on protein investment as well as insights into its regulation. Here, we present an inter-laboratory, standardized, multi-omics study of the metabolic shift in L. lactis, carried out in the light of the predictions of the self-replicator model. First, we investigate the metabolic shift at multiple cellular levels, and second, we examine growth rate-related regulation in L. lactis. We study the metabolic shift by focussing on the energy generating pathways of L. lactis: glycolysis and arginine metabolism and also investigate the ribosomal protein investment.
Results

The metabolic shift and bioenergetics

In order to study the classic metabolic shift from mixed acid fermentation (production of formate, acetate and ethanol) to homolactic fermentation, i.e. production of lactic acid in \textit{L. lactis} in glucose-limited chemostat cultures at varying growth rates, we set the dilution rates (D, henceforth referred to as growth rate) accordingly to 0.15 h\(^{-1}\); 0.3 h\(^{-1}\), 0.5 h\(^{-1}\) and 0.6 h\(^{-1}\), but measured the actual dilution rate on the day of harvesting. This resulted in a slightly different dilution rate for some chemostat cultures. The largest deviation was found for one chemostat, which ran at a D of 0.45 h\(^{-1}\) while being set at a D of 0.5 h\(^{-1}\) (Table 1). For transcriptomic and proteomic analysis, the deviation did not result in an outlier; the samples derived from this chemostat still contribute to the triplicate measurement. A growth rate of 0.6 h\(^{-1}\) is close to the maximal growth rate in this medium, and a growth rate of 0.15 h\(^{-1}\) is expected to mainly yield products of mixed acid fermentation. Indeed, \textit{L. lactis} displayed a rather steep metabolic shift at increasing growth rates (Fig. 1A). The fraction of lactate flux (normalized to the total carbon flux) increased at higher growth rates, from about 10\% to 75\%. The decreasing mixed acid branch flux and increasing lactate flux overlapped between growth rates 0.3 and 0.5 h\(^{-1}\). Energetically, homolactic fermentation generates 2 ATP per glucose, while mixed-acid fermentation generates 3 ATP per glucose. Yet, a 65\% increase in lactate flux corresponding to a 22\% decrease in ATP production was not accompanied by a drastic decrease in biomass concentration, which decreased only 14\% at D = 0.6

![Figure 1](image-url)

**Figure 1.** (A) Fraction of total carbon flux towards homolactic and mixed-acid branches. (B) Total ATP formation rates calculated by substrate-level phosphorylation (\(V_{\text{total, SLP}}\)), by the genome-scale stoichiometric network model (\(V_{\text{total, GS}}\)), and the maximum possible ATP production rate (\(V_{\text{ATP}}\)).
71 h\(^{-1}\) (homolactic metabolism) compared with D = 0.15 h\(^{-1}\) (mixed-acid metabolism) (Table 1). The catabolic and total carbon balances were closed with less than 17% standard deviation. We calculated the ATP formation rates by substrate-level phosphorylation (V\(_{\text{total, SLP}}\)) and also via the genome-scale stoichiometric network model (Flahaut, et al., manuscript submitted), (V\(_{\text{total, GS}}\)) and the maximum possible ATP production rate (v\(_{\text{ATP}}\)) (Fig. 1B). The curve V\(_{\text{total, SLP}}\) plateaus above D = 0.5 h\(^{-1}\). Thus, at D = 0.6 h\(^{-1}\) cells grow faster than at D = 0.5 h\(^{-1}\), but at the same rate of ATP formed per unit biomass. The curve V\(_{\text{total, GS}}\) is steeper, but also plateaus. The maximal ATP formation rate increases linearly to D=0.5 h\(^{-1}\) but remains stable at the highest growth rate of D = 0.6 h\(^{-1}\).

Fluxes do not relate to V\(_{\text{max}}\), protein- or transcript abundance

We obtained fluxes by averaging the flux ranges predicted by flux variability analysis (FVA) on the genome-scale model of \(L.\ lactis\) MG1363 (Fig. 2). The glycolytic flux linearly increased with growth rate. The flux through the homolactic-branch enzyme

<table>
<thead>
<tr>
<th>Dilution rate (h(^{-1}))</th>
<th>Biomass (gDW.L(^{-1}))</th>
<th>Catabolic C balance %(^{ab})</th>
<th>C balance %(^{abc})</th>
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<tbody>
<tr>
<td>0.15</td>
<td>0.803 ± 0.068</td>
<td>81.02 ± 8.24</td>
<td>100.3 ± 9.84</td>
</tr>
<tr>
<td>0.15</td>
<td>0.797 ± 0.116</td>
<td>84.41 ± 14.1</td>
<td>103.5 ± 16.7</td>
</tr>
<tr>
<td>0.15</td>
<td>0.826 ± 0.017</td>
<td>86.45 ± 3.82</td>
<td>106.2 ± 4.54</td>
</tr>
<tr>
<td>0.3</td>
<td>0.842 ± 0.097</td>
<td>83.48 ± 10.9</td>
<td>103.7 ± 13.1</td>
</tr>
<tr>
<td>0.3</td>
<td>0.806 ± 0.105</td>
<td>79.14 ± 11.6</td>
<td>98.56 ± 13.9</td>
</tr>
<tr>
<td>0.3</td>
<td>0.840 ± 0.029</td>
<td>77.39 ± 3.58</td>
<td>97.53 ± 4.34</td>
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<tr>
<td>0.5</td>
<td>0.762 ± 0.023</td>
<td>84.02 ± 4.28</td>
<td>102.3 ± 4.77</td>
</tr>
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<td>0.790 ± 0.074</td>
<td>85.96 ± 9.97</td>
<td>104.9 ± 11.5</td>
</tr>
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<td>0.5</td>
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<td>79.29 ± 4.13</td>
<td>96.59 ± 4.60</td>
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<td>0.6</td>
<td>0.734 ± 0.005</td>
<td>72.84 ± 2.74</td>
<td>90.44 ± 2.95</td>
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<tr>
<td>0.61</td>
<td>0.719 ± 0.002</td>
<td>85.98 ± 3.61</td>
<td>107.85 ± 4.05</td>
</tr>
<tr>
<td>0.613</td>
<td>0.641 ± 0.008</td>
<td>81.85 ± 5.03</td>
<td>102.51 ± 5.95</td>
</tr>
</tbody>
</table>

a % C-Balance = \(\% (q\text{C-out} / q\text{C-in})\); C-moles: glucose=6, lactate=3, pyruvate=3, ethanol=2, acetate=2, succinate=4, biomass=27.8 gDW/C-mole
b Excluding biomass
c Including biomass
V max and fluxes in mmol.gdw−1.hour−1. In all metabolic fluxes, by averaging the flux maps predicted by flux variability analysis (FVA) on the genome-scale model of L. lactis MG1363, constrained by all measurements of nutrient consumption and product formation rates. Gray areas represent standard error. ▲ AckA1, POM transcript shows lmg0355 (gpmA), ADH transcript shows adhE.
lactate dehydrogenase (LDH) increased nonlinearly, and fluxes through the mixed-acid-branch enzymes pyruvate formate lyase (PFL), acetate kinase (ACK) and alcohol dehydrogenase (ADH) increased at \( D = 0.3 \text{ h}^{-1} \) and then decreased further on. In contrast to these changes in fluxes, the \( V_{\text{max}} \)'s, protein- and transcript ratios altered very little. (Fig. 2, supplementary material, Table S1 and S2).

The \( V_{\text{max}} \)'s and protein ratios of all glycolytic enzymes remained more or less constant up to \( D = 0.5 \text{ h}^{-1} \) except for PFL, of which the protein ratios decreased linearly with a growth rate above \( 0.15 \text{ h}^{-1} \). Between \( 0.5 \text{ h}^{-1} \) and \( 0.6 \text{ h}^{-1} \), the enzymes encoded by the \( las \) operon, phosphofructokinase (PFK), pyruvate kinase (PYK) and LDH showed increases in \( V_{\text{max}} \)'s and protein ratios. Apart from these enzymes, phosphoglucone isomerase (PGI), fructose bisphosphate aldolase (ALD), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK) and phosphoglycerate mutase (PGM) all showed a rise in \( V_{\text{max}} \) value at \( D = 0.6 \text{ h}^{-1} \). Of these, ALD and TPI also showed a rise in protein ratios. Glucokinase (GLK) \( V_{\text{max}} \) showed a gradual decrease overall, while the GLK protein ratio increased at \( D = 0.6 \text{ h}^{-1} \). The enzymes involved in the mixed-acid-fermentation pathway showed a decreasing trend. Phosphotransacetylase (PTA) protein ratios, and ADH and ACK \( V_{\text{max}} \) and protein ratios decreased at \( D = 0.6 \text{ h}^{-1} \). The two copies of acetate kinase showed antagonistic behaviour. At higher growth rate, the \( ackA1 \) transcript increased while the protein ratio was constant, whereas the \( ackA2 \)

![Image](image.png)

**Figure 3.** (A) Correlation between \( V_{\text{max}} \) and protein \( \log \) regulation ratios of all glycolytic and fermentative enzymes at various growth rates. Enzyme activities for which the regulation ratio deviates by at least 1 in one of the measurements are coloured. (B) Metabolic regulation coefficients for different growth rate pairs.
transcript was constant while the relative AckA2 protein ratio decreased to a third compared with that at 0.3 h\(^{-1}\). The correlation between enzyme activities and their respective protein ratios as measured in the proteome studies are shown in Fig. 3A. Except for GLK, ACK and ADH, most enzymes lie on the perfect correlation line with a slope of 1 denoting excellent correspondence between regulation of protein ratios and regulation of enzyme activities, but overall, the changes in \(V_{\text{max}}\) and protein ratios do not correlate proportionally with the changes in fluxes.

At the transcript level, \(pgiA\) (PGI), \(fbaA\) (ALD) and \(gpmA\) (PGM) were linearly activated with growth rate (Fig. 2). Glycolysis in \(L. lactis\) is regulated at different levels by a variety of mechanisms. One of the predominant ones is carbon catabolite repression (CCR) orchestrated by carbon catabolite control protein CcpA\(^{27,28}\). The number of significantly regulated transcripts of genes of the CcpA regulon increased, as well as their fold-changes, with higher growth rate (supplementary material, Table S1). Genes displaying CCR have an upstream binding motif (\(cre\)-site) to which the CcpA-HPr Ser46-P complex can bind\(^{27,29}\). An important part of the CcpA regulon is the \(las\) operon. The transcriptional activity of \(ldh\), \(pyk\) and \(pfk\) is enhanced upon binding of the CcpA complex in the \(las\) promoter region\(^{30}\). Even though at high growth rates CCR is functional both at the transcriptional and protein level, no coherent influence of CcpA on the transcription of glycolytic genes is seen. For instance, at a growth rate of 0.6 h\(^{-1}\) only the \(pyk\) gene showed a significant transcriptional increase, while transcription of \(ldh\) and \(pfk\) genes did not change. Thus, overall we did not observe many significant changes in transcription of genes encoding glycolytic enzymes, except for a few (\(but-AB\) and \(pgiA\)) at the highest growth rate of 0.6 h\(^{-1}\) (supplementary material, Table S1).

**The metabolic shift is predominantly regulated at the enzyme activity level**

To investigate the growth rate-related flux regulation in \(L. lactis\), we used regulation analysis\(^{31}\) for a quantitative analysis of the control of glycolytic and fermentative fluxes with increasing growth rate. The hierarchical regulation coefficient (\(\rho_h\)) represents the extent of flux regulation through gene expression and via changes in enzyme concentration. It can be defined as:

\[
\rho_h = \frac{\Delta \ln V_i}{\Delta \ln e_i} \times \frac{\Delta \ln e_i}{\Delta \ln J} = \frac{\Delta \ln e_i}{\Delta \ln J}
\]

for a pathway flux \(J\), with concentration \(e_i\) of enzyme i which carries a flux at a rate \(V_i\). The coefficient \(\rho_h\) for a set of two dilution rates was thus calculated as the ratio of
the difference in the logarithm of the fluxes at both dilution rates to the difference in
the logarithm of the enzyme activity. The metabolic regulation coefficient ($\rho_m$) repre-
sents the extent of flux regulation as a result of metabolic regulation of enzyme activ-
ity, defined as

$$\rho_m = \sum \frac{\partial \ln V}{\partial \ln x} \times \frac{\partial \ln x}{\partial \ln J}$$

At steady state, the sum of the regulation coefficients $\rho_h$ and $\rho_m$ is one. In our data,
hierarchical regulation coefficients of the glycolytic and fermentative pathways are
close to zero (Fig. 3B) because the flux increases substantially, while the $V_{max}$ remains
more or less unchanged except for the change in D from 0.5 h$^{-1}$ to 0.6 h$^{-1}$, where the
$V_{max}$ values increase. In other words, the metabolic coefficients are close to 1, repre-
senting the constant maximal enzyme activity over the change in glycolytic flux and
the metabolic shift. All $\rho_m$'s between D = 0.15 h$^{-1}$ and 0.5 h$^{-1}$ are above 0.8. Between
D = 0.3 h$^{-1}$ and 0.6 h$^{-1}$, $\rho_m$'s are lower, indicating partial hierarchical regulation, and
from 0.5 h$^{-1}$ to 0.6 h$^{-1}$, except for glyceraldehyde phosphate dehydrogenase (GAPDH),
PTA and ACK, the $\rho_m$’s are zero, indicating complete hierarchical regulation. ADH is
hierarchically regulated at all growth rate-pairs except for 0.15 h$^{-1}$ to 0.5 h$^{-1}$.

**Ribosome investment**

One of the predictions of the self-replicator model $^{22}$ is the proportional increase in
investment in ribosomal protein (rProtein) with increasing growth rate. The largest
fraction of lactococcal RNA consists of ribosomal RNA (rRNA). In *E. coli*, rRNA is
approximated to amount to 85% of the total RNA (totRNA) in the cell $^{32}$. The total
amount of RNA divided by the total amount of protein (totProt) is an accepted method
to calculate the ribosomal content of a cell. We therefore quantified totRNA and totProt
for a fixed cell density for all chemostat samples. The totRNA/totProt ratio increased
linearly with, but not proportionally to the growth rate, and its increase relative to the
lowest growth rate levels off at the highest growth rate (Fig. 4). For the transcript and
protein ratios of rProteins, a very small increase is seen relative to that of the lowest
growth rate (Fig. 4). The rProtein ratios show a somewhat steeper increase at increas-
ing growth rate than the mRNA ratios of rProteins (Fig. 4). The largest increase is
observed in the ratio of totRNA/totProt, an indication for the amount of rRNA (Fig. 4).
Amino acid consumption

To better understand the overall metabolism of *L. lactis*, we also quantified the amino acid consumption rates (supplementary material, Fig. S1). The consumption rates of most amino acids steadily increased with the increase in the growth rate. Aspartate and glutamate measurements could not reliably be determined as the concentrations were too close to the detection limit. The production of ornithine, citrulline and ammonia steadily increased with concomitant consumption of arginine (Fig. 6, supplementary material, Fig. S1), which changed in a bow-like fashion with growth rate. At 0.15 h⁻¹, arginine was consumed at a rate of 0.28 mmol·gdw⁻¹·h⁻¹; this consumption rate gradually increased up to 0.8 mmol·gdw⁻¹·h⁻¹ at a growth rate of 0.5 h⁻¹. Subsequently, however, at growth rate 0.6 h⁻¹, the consumption rate dropped to a value lower than that at growth rate 0.15 h⁻¹. The same pattern was observed at the transcript and protein ratios of the responsible proteins ArcABC1C2D1D2. An initial increase in the expression

Figure 4. Change in totRNA/totProt ratio (A), mRNA ratios of rProteins (B) and rProtein ratios relative to the lowest growth rate 0.15 h⁻¹, for *L. lactis* (C) (this study) and relative rProtein ratios from the proteome data of *E. coli* (D) 33.
ratios of the genes arcAC1C2D1 was followed by a significant reduction of the same transcripts plus arcB when comparing dilution rates 0.5 h⁻¹ and 0.6 h⁻¹. At the protein level, ArcABC2 showed a similar trend. The ArcA protein ratio did not change between 0.15 h⁻¹ and 0.5 h⁻¹, a decrease was only seen at 0.6 h⁻¹.

**Fatty acid biosynthesis**

Another module in the self-replicator model is that of lipid biosynthesis as an essential part of membrane biogenesis. The composition of acyl chains in the phospholipids of lactococcal membranes was investigated at varying growth rates. Most of the fatty acid biosynthesis genes are organised in one large operon in *L. lactis*, and are regulated by FabT (this thesis, Chapter 4). Transcription of these genes does not change coherently with increasing growth rate (supplementary material, Table S1). Only at the highest growth rate transcription of *fabZ1* (*llmg0538*) is upregulated significantly. The dehydratase FabZ1 in *Enterococcus faecalis* is known to function as an isomerase that decreases the acyl-chain saturation ratios. Thus, we determined the length and degree of saturation of the acyl chains of the lactococcal cell membrane at different growth rates (Fig. 5). At the lowest growth rate, the cell membranes contain more saturated acyl chain (66%) than at the other growth rates. Also, the short C14 and C16

![Figure 5. Acyl chain composition analysis with increasing growth rate. Saturated acyl chains are shown in blue, while unsaturated acyl chains are shown in red. Lighter colours indicate longer acyl chains. Shown are the averages of three biological experiments.](image)
Figure 6. (A) Schematic overview of the arginine metabolic pathway in relation to the relative transcription and protein ratios and the metabolic fluxes per enzyme. With the available omics data fluxes were generated; empty graphs indicate that no significant data was available. (B) Representation of the different repression mechanisms that balance arginine metabolism. At low glucose concentrations, ArgR (homohexamer) blocks the catabolic arc-operon at low arginine levels. At high arginine levels, ArgR and AhrC form a heterohexameric complex that blocks the anabolic arg-operon. At high glucose concentrations, repression of the arc genes at high arginine levels is taken over by carbon catabolite repression through CcpA/HPr-Ser-P.
Acyl chains are more abundant at the lowest growth rate (69%). Both the saturation rate and the contribution of short acyl chain lengths decrease significantly when the growth rate increases. At the highest growth rate the degree of saturation is 54% and the percentage of small acyl chains is 55%.

**Growth rate-related stress**

Proteins and their transcripts related to stress were obtained from the GO-database (Table 2) 36. Most stress-related transcript and protein ratios remain unchanged between the growth rates of 0.3 h\(^{-1}\) and 0.5 h\(^{-1}\), while most changes, if any, take place between growth rates of 0.5 h\(^{-1}\) and 0.6 h\(^{-1}\) (Fig. 7A). The correlation between transcripts and proteins for this class is relatively high with a \(r\) of 0.632 (Fig. 7B).

**List of putative isoenzymes**

The proteome of *L. lactis*, as obtained by the annotation of the genome sequence contains a number of isoenzymes that could effectively perform the same function. This feature is not unique to *L. lactis*, since it is prevalent in a multitude of organisms. The possible reason for having isoenzymes is that it offers flexibility of regulation under a wide variety of environmental conditions 37. We classified all the observed proteins of our dataset according to their annotated enzyme functions to compile a “putative”

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Enzyme Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Llmg0080</td>
<td>osmotically inducible protein C</td>
</tr>
<tr>
<td>Llmg0093</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>Llmg0638</td>
<td>ATP-dependent Clp protease</td>
</tr>
<tr>
<td>Llmg0986</td>
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<td>universal stress protein A2</td>
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<td>glutathione peroxidase</td>
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<td>Llmg1350, Lmg1351, Llmg1352</td>
<td>putative tellurium resistance protein</td>
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<tr>
<td>Llmg2302</td>
<td>non-heme iron-binding ferritin</td>
</tr>
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</table>

Table 2. Stress related proteins from GO annotation, as determined by EBI genome reviews.
isoenzyme list of 115 enzyme groups (supplementary material, Table S3). In about half of the enzyme groups, proteins were not detected in our samples. Next to that, there was considerable variation in the spectral counts of the detected peptides. This variation might indicate which proteins are more prominently involved in carrying out the respective reactions under glucose-limited chemostat growth of \textit{L. lactis}. When a protein is undetected by our experimental setup this does not confirm its absence, but it does indicate that such protein may be present at very low quantities. This kind of information can be useful for (iso)enzyme studies with respect to engineering metabolic pathways under specific environmental conditions.

\textbf{Figure 7.} (A) Stress-related proteins and their transcript ratios at increasing growth rate. Gray bars indicate the standard deviation. (B) Correlation between stress-related transcript and protein ratios.
Discussion

Experimental design
In this study we characterized the metabolic shift from mixed-acid to homolactic fermentation in the model lactic acid bacterium *L. lactis*. We designed our experimental setup, and based it on the predictions of the self-replicator model, to test the hypothesis that shifts in metabolic strategies are outcomes of evolutionary optimization of resource allocation. The proteome data show that a major fraction of total protein is invested in glycolysis and ribosome synthesis (supplementary material, Table S2). To quantify the protein investment in *L. lactis*, we measured the maximal enzyme activities and relative protein levels. To investigate possible regulation in protein investment, genome-wide responses in transcription were examined. We characterized the metabolic shift by also measuring extracellular metabolites. Intracellular fluxes were inferred by variability analysis on the genome-scale model, taking the average values to depict each flux.

There are no studies characterising the metabolic shift of *L. lactis* at the multi-level scale and only a few studies investigated the response of growth rate on changing environmental conditions. Of these, one focussed on amino acid metabolism in *L. lactis* strain IL1403 by growing chemostat cultures under isoleucine limitation. This experimental design resulted in glucose excess in all chemostats. Since glucose is a preferred substrate, glucose repression was constantly on in this study and the metabolic shift was not observed. Another study did look at glucose limitation by growing the same strain in accelerostats, but the metabolic shift to mixed-acid fermentation was not observed at the lower growth rates. *L. lactis* strain ML3, an ancestor from MG1363, is known to exhibit the metabolic shift at different growth rates in glucose-limited chemostats. Therefore, we characterized the glucose-limited, growth rate-mediated metabolic shift in *L. lactis* and employed inter-laboratory standardized protocols. A graphical representation of the experimental setup is shown in Fig. 8.

Lack of correlation between transcriptomics and proteomics data
The correlation between the data of the transcriptome and the proteome experiments was generally low with $r = 0.262$. When looking at individual functional categories, for instance the CcpA regulon, a linear dependence is seen for all significantly altered
transcripts and proteins with $r = 0.536$ (supplementary material, Table S4). Similar results were obtained for several other pathways represented in the KEGG database, with the highest correlation coefficients being 0.61 for starch and sucrose metabolism and 0.56 for glycine, serine and threonine metabolism. All other pathways had lower linear dependencies or contained less than 10 genes (supplementary material, Table S4). Altogether the change in protein ratios is not proportional to the change in transcript ratios. In a recent chemostat study, in which the growth rate of one culture of L. lactis was gradually increased, the correlation between data of transcriptome and proteome was up to 0.69\textsuperscript{39}. Using a similar experimental setup, comparable correlations were seen between the transcriptome and proteome of E. coli\textsuperscript{33}. In independent chemostat cultures of Saccharomyces cerevisiae, for instance, transcriptional changes do not largely contribute to glycolytic behaviour\textsuperscript{11,41}. It is more likely that the glycolytic fluxes are regulated at the post-transcriptional level, partly explaining the poor correlation between gene transcription ratios and protein expression ratios. Other studies in yeast show that genes with a high correlation between their output at the transcriptomic and proteomic level are often adaptive. Examples are genes involved in stress-response and usually not genes encoding proteins involved in core metabolic functions like glycolysis\textsuperscript{42,43}. The fact that a correlation between mRNA and protein ratios is lacking for glycolytic enzymes suggests that an mRNA-buffer exists for glycolysis genes in L. lactis. Indeed, protein turnover and mRNA lifetimes are important posttranscriptional aspects playing key regulatory roles\textsuperscript{44,45}.

**Correlation of fluxes with proteomics: overcapacity of enzymes over flux**

The glycolytic flux increases proportionally with the growth rate. However, this is not the case for transcripts, proteins and enzyme activities. In fact, the ratio of $V_{\text{max}}/\text{flux}$ shows that the enzyme activities are much higher than the actual flux inside the cell at all growth rates for all enzymes except for PGM, GAPDH and enolase (ENO) (Fig. 2). The overcapacity of enzymes suggested by the $V_{\text{max}}/\text{flux}$ ratios can explain the unchanged enzyme levels in spite of a four-fold increase in the flux. Therefore, it is not surprising to see metabolic regulation coefficients with a value of 1 because the increase in flux might simply be because of a higher glucose concentration resulting in enzymes with higher flux. What is most surprising is that the enzymes of the mixed-acid branch and the lactic-acid branch also show an overcapacity. We cannot, however, ignore the possibility that enzyme activities were measured *in vitro* and might over-
estimate in vivo \( V_{\text{max}} \)'s. GAPDH, PGM and ENO are exceptions because of technical issues; as detailed in the Materials and Methods section, GAPDH and PGM activities were determined by old sub-optimal enzyme assays since the in vivo-like assay medium specifically designed for this study had not been finalized yet. The activity of ENO is sensitive to ammonium sulphate, present in the buffer because of adding coupling enzymes that are suspended in ammonium sulphate solution. PFL activity could not be measured, as the enzyme is prone to oxidation. It was shown before that protein ratios of PFL is a determining factor for the metabolic shift from mixed-acid. Indeed we see PFL protein ratios go down with increasing growth rate (Fig. 2).

**Correlation of \( V_{\text{max}} \) with protein ratios**

One would normally expect a good correlation between the amount of an enzyme and the activity of that enzyme because the \( V_{\text{max}} \) is a product of total enzyme concentration and the catalytic turnover number \( (k_{\text{cat}}) \). Of course, this is only true, when post-translational modification affecting the enzyme activity does not occur. When looking at the correlation of \( V_{\text{max}} \) and the respective protein ratio for all measured enzymes (Fig. 3A), we found that GLK, ACK and ADH do not lie on the perfect correlation line (slope = 1). This might indicate that these enzymes undergo post-translational modification resulting in differential regulation of enzyme activity without much change in the protein level. Post-translational modification of proteins is abundant in eukaryotes as well as prokaryotes. A recent study revealed phosphorylation of proteins at the amino acid residues serine, threonine and tyrosine in a strain of *L. lactis*. The importance of the post-translational modification as a regulatory mechanism has generated increased attention and a database of phosphorylated proteins (PHOSIDA) has recently been developed.

It is interesting to note that of the enzymes that do not perfectly correlate with their protein ratios, one is involved in phosphorylating glucose and two (ACK and ADH) are involved in the metabolic shift. Although surprising at first, it seems logical that the enzymes of the mixed-acid branch (ACK, ADH) show indications of post-translational modification, because they are important in determining the metabolic flux in either direction of the metabolic shift in *L. lactis*. It is unclear why GLK appears in this database of phosphorylated proteins. *L. lactis* predominantly uses PTS systems for glucose transport, while GLK is only useful when a glucose permease (GlkU) is used. There could be two explanations for this phenomenon. One, GLK might un-
dergo post-translational modification as its need will most likely rise at fast growth. Second, we might be missing an activator for GLK during $V_{\text{max}}$ assay measurements with resultant underestimated GLK $V_{\text{max}}$'s.

The increase of ribosomes is not proportional to the increase in growth rate

In order to define the relationship between growth rate and ribosomal content in *L. lactis*, we characterized the amount of both ribosomal RNA and total protein for each growth rate. The ratio of totRNA over totProt is proportional to growth rate can be equated with the proportionality of ribosome abundance with growth rate. Since, an increase in growth rate demands a higher mRNA translation capacity of the cell. Gausing reported that a rise in the growth rate of *E. coli* is concomitant with an increase in rProtein synthesis. From a specific growth rate of 0.6 h$^{-1}$ to 2.2 h$^{-1}$ this increase is even proportional. In other words, doubling the growth rate of *E. coli* also doubles the amount of ribosomes. In our experiments we observed a gradual, albeit non-proportional increase in ribosomal content with increasing growth rate. This was for a major part caused by a steep rise in the amount of rRNA accompanied by only a slight increase in the rProtein pool with increasing growth rates. If we take into account that a rather stable level of mRNA of most of the related rProtein yields more synthesized protein at higher growth rates, our data indicate a restrained yet efficient strategy for total ribosomal content of a lactococcal cell. At all growth rates, a certain amount of mRNA for rProteins exists that can be used by the cell to synthesize the required amount of rProteins, as demanded by the growth rate.

The results presented here support the idea that the major limitation for an increase in the ribosomal content is the synthesis of rRNA, as shown by the upwards linear trend of the totRNA/totProt ratio (Fig. 4A). Even when the rProteins are limiting at high growth rates, this does not necessarily mean that ribosome activity is affected. First of all, most rProteins are located on the outside of the ribosome, and do not play a direct role in protein synthesis. Secondly, part of the rProteins can be removed without a loss in ribosome activity, which then points toward the idea that rRNA as such is indeed functional as a ribozyme without the requirement of rProteins.

The relationship between ribosome abundance and ribosome synthesis rate is not obvious. In the assembly of a ribosome, the early-assembly rProteins are thought to structure the rRNA in such a way that it functions as a ribozyme. In our dataset the amount of early-assembly rProteins shows a gradual increase with increasing growth
rate (supplementary material, Fig. S2), but it is not proportional to the rRNA synthesis rate as derived from the totRNA/totProt ratio (Fig. 4A). So even the minimally required subset of rProteins does not follow the same trend as the rRNA production rate with an increasing growth rate. In the proteomic dataset of \textit{E. coli}, growing at different growth rates\textsuperscript{33}, the relative ratios of rProteins show trends comparable to those in our study for the rProtein synthesis rate (Fig. 4C and D). Since not many available datasets provide this type of rProtein abundances from cells growing at varying growth rate, it is too early to speculate on a general phenomenon for bacteria. However, we seem to have found that at increasing growth rates ribosomes are less decorated with rProteins than at lower growth rates.

\textit{L. lactis} strategy to avoid degradation of ribosomes at low growth rates

The fact that at a low growth rate the ratio of rRNA/rProt is much lower than at high growth rates strengthens the idea that when the growth rate decreases excess rRNA is degraded until the moment where the rProteins prevent total breakdown of the ribosome. In \textit{E. coli}\textsuperscript{59} and \textit{Staphylococcus aureus}\textsuperscript{60} it has been shown that ribosomes tend to dimerize when the cells reach stationary phase. Because detached ribosomal subunits are the actual targets of ribonuclease activity\textsuperscript{61}, dimerized ribosomes have an increased protection against RNA degrading enzymes. The ribosome dimerizing factors in \textit{E. coli} possess a mutual homology with the YfiA protein in \textit{L. lactis}. The level of \textit{yfiA} transcripts is downregulated when growth rate increases. We have shown recently that \textit{L. lactis} YfiA is involved in ribosome dimerization when the cells enter the stationary phase (this thesis, Chapter 5). Dimerization of ribosomes by YfiA is seen as a possible strategy for \textit{L. lactis} when, at lowest growth rates, part of the ribosome pool is protected against degradation. Since the cells conserve their ribosomes at lower growth rate, they allow a quick recovery of translation, and thus of growth, as soon as the cells encounter new resources in the environment.

Arginine metabolism as a function of the growth rate

Arginine serves as a source of carbon and nitrogen in \textit{L. lactis}. This strain can catabolize arginine to obtain an additional ATP via the arginine deiminase (ADI) pathway\textsuperscript{62,63}. The concomitant production of ammonia leads to less acidification of the environment\textsuperscript{64}. Arginine degradation via ArcA produces citrulline, which can be further catabolized by the ornithine carbamoyltransferase ArcB either to carbamoyl-P or or-
nithine. ArcC then degrades carbamoyl-P into ammonia and carbon dioxide, producing 1 mole of ATP per mole of arginine. Ornithine is exchanged for arginine by the arginine-ornithine antiporter ArcD1/2. The transcriptomic- and proteomic analyses reveal that up to a growth rate of 0.5 h\(^{-1}\) arginine catabolism increases, after which a steep decrease occurred at a growth rate of 0.6 h\(^{-1}\), leading to a strongly reduced amount of arginine catabolic enzymes and their transcripts. This was confirmed by the consumption rates of arginine and the production rates of ornithine, citrulline and ammonia. The upstream region of the arginine catabolic gene cluster arcABD1C1C2TD2 contains 6 ARG boxes for binding of ArgR, a putative CodY operator site and a cre-site for CcpA binding. At high levels of arginine, the two arginine regulators ArgR and AhrC derepress the ADI pathway. With increasing growth rates, arginine degradation could function as a glycolysis-independent system for ATP generation. However, at a growth rate of 0.6 h\(^{-1}\) arginine might be required for biomass production, which would, reduce the intracellular arginine level and repress the arginine catabolic arc operon (Fig. 5). If the arginine concentration were indeed low at a high growth rate, a transcriptional response of the arginine biosynthetic genes argCJBF, argGH and gltSargE might be expected. However, no significant upregulation of arginine biosynthesis is seen. It is therefore more likely that CcpA repression causes the steep decrease in ADI activity as at near-maximal growth rates the amount of residual glucose quickly increases (Fig. 5B), which would lead to carbon catabolite repression of the arc operon.

**Behaviour at near-maximal growth rate**

The limits of growth were approached in the condition with D = 0.6 h\(^{-1}\). We suppose that *L. lactis* MG1363 cannot grow much faster under these conditions, and with the growth rate employed here cells were approaching “a washout”. The biomass went down at highest growth rates (Table 1), and the concentration of residual glucose in the chemostats changed quite drastically from undetectable in the chemostats at D = 0.15 h\(^{-1}\) up to 0.5 h\(^{-1}\) to a few mM at the highest dilution rate, 0.6 h\(^{-1}\). The medium was supplemented with all components up to concentrations that only glucose was the limiting factor. The amounts of some stress proteins went down at the highest growth rate. This is reminiscent to the feast / famine kind of behaviour seen in *B. subtilis* and *E. coli*, which comprises cells being prepared for all kinds of stresses at low growth rate and then suddenly investing much less in stress machineries when they...
encounter high glucose concentrations, at higher growth rates. The transcript ratios of the CcpA and HPr encoding genes increased significantly with a rising growth rate (supplementary material, Table S1). Importantly, the phosphorylation state of HPr, i.e. HPr-Ser-P, increases with growth rate and this may directly impact the glycolytic and other metabolic rates \(^{70,71}\). Both the transcriptome and proteome are significantly, but not drastically, reorganized upon the growth rate at 0.6 h\(^{-1}\) (supplementary material, Table S1 and S2). Altogether, the impact of growing at a growth rate close to maximal as compared to lower growth rates yield mild differences in the proteome and the respective transcriptome of \textit{L. lactis}.

**Why does \textit{L. lactis} behave differently, compared to other microorganisms?**

As has been detailed above, \textit{L. lactis} maintains an almost identical proteome throughout a wide range of growth rates, which is, to say the least, non-optimal in view of protein costs. An assumption of the self-replicator model is that there is a choice between a metabolically (energetically) efficient (high-yield) and kinetically efficient (high-rate) pathway. In \textit{L. lactis} the mixed-acid route is analogous with the former and the lactate route with the latter. From our data it seems that \textit{L. lactis} fermentation type is largely determined by effectors that change in response to external factors. When \textit{L. lactis} is growing fast, the level of determining factors like PFL protein ratios is reduced (Fig. 2), thereby reducing the inhibition of the lactate flux \(^{48}\). Another candidate for regulation of LDH activity is fructose-bis-phosphate (FBP) \(^{72}\). The flux of FBP formation increases linearly with growth rate (Fig. 2) \(^{73}\). Both the decrease of PFL protein levels and the increase for FBP formation flux confirm the observations from earlier experiments. When the growth rate increases, a shift towards lactate fermentation takes place under the influence of both the reduced protein level (PFL) and an increase of FBP at high growth rates \(^{73}\). Despite our multi-omics approach we can however not exclude that other glycolytic intermediates and/or unknown factors play an important role in the metabolic shift from mixed-acid to homo-lactic fermentation.

Another inherent assumption of the self-replicator model is that organisms are evolutionarily optimized, and in this optimal state, differential protein allocation results in a metabolic shift. If this assumption of evolutionary optimization were true, then the actual test of the hypothesis would be via long-term evolution experiments. In such a case one explanation for the non-conformation of \textit{L. lactis} to the protein economy hypothesis is that it might not be evolutionarily optimized for the conditions tested in
the laboratory. Native to a rich environment of milk, this microorganism might have been selected for growth on high sugar concentrations, always facing enough substrate to support heavy investments in protein. The cost of using resources scantily and not being able to use an unexpected abundance of nutrients might in fact be penalizing in a rich substrate environment in the presence of many competitors.

Materials and methods

Strain and growth medium

*Lactococcus lactis* ssp. *cremoris* MG 1363 was grown on chemically defined medium for prolonged cultivation (CDMPC) as described by Santos et al., (manuscript in preparation) with 25 mM glucose as the limiting nutrient and the medium composition as detailed before.

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% N₂ (99.998% pure) and 5% CO₂ (99.7% pure) with oxygen impurity less than 34 vpm. A pH of 6.5±0.05 was maintained by automatic titration with 5 M NaOH. Fermenters were inoculated with 4% (v/v) of standardized precultures consisting of 45 mL of CDMPC inoculated with 300 µL of a glycerol stock of *L. lactis* MG 1363 and incubated for 16 h at 30°C. After batch growth until an optical density at 600 nm (OD₆₀₀) of around 1.8, medium was pumped at the appropriate dilution rate (0.15, 0.3, 0.45, 0.5, 0.6 h⁻¹).

Harvesting of cells from chemostats

The chemostats were harvested assuming a steady state at 10 working volume changes. 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 performed in triplicate for each sample as follows. 4 mL of culture was filtered through a pre-dried, pre-weighed 0.2 µm cellulose nitrate filter (Whatman GmbH, Dassel, Germany), washed twice with deionized water and dried to a constant weight. For one unit change of optical density, the change in dry weight was determined to be $0.31\pm0.02 \text{ g.L}^{-1}.\text{OD}_{600}$.

Fermentation end-product, ammonia and amino acid analysis
Supernatant samples from medium bottles and chemostat fermentations were prepared by filtering through a 0.20 µm polyethersulfone (PES) filter (VWR international B.V., Amsterdam, the Netherlands) and storing the flow-through at -20ºC until further analysis. Extracellular concentrations of lactate, acetate, ethanol, formate, and glucose were determined by High Performance Liquid Chromatography (HPLC) on a Shimadzu (Tokyo, Japan) LC-10AT liquid chromatograph equipped with a Shimadzu (Tokyo Japan) RID-10A refractive index detector for ethanol and glucose, and a Shimadzu (Tokyo, Japan) SPD-10A VP UV-Vis absorbance detector set at 210 nm for the remaining metabolites. Separation was carried out on a Bio-Rad Aminex Ion exclusion HPX-87H column equilibrated at 55ºC with an isocratic flow of 5 mM H$_2$SO$_4$ set to 0.5 mL · min$^{-1}$. The injection volume used was 50 mL and concentrations were estimated in triplo by comparison of peak areas to a calibration curve obtained with standards analyzed under the same conditions. Residual glucose concentrations were determined by enzymatic coupling with NADP$^+$ in an assay containing 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid)-KOH, 5 mM MgSO$_4$, 2 mM ATP, 4.5 mM NADP$^+$, 1.5 U·mL$^{-1}$ hexokinase, 1 U·mL$^{-1}$ 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). Amino acid vials were prepared in 950 µL volumes by adding 25 µL each, of 0.1 M Borate, 1 mM of Norvaline as an internal standard, and culture supernatant samples, and the rest, milli-Q water. Amino acid concentrations were determined with an AminoQuant 1090 High Performance Liquid Chromatography (Shimadzu, Kyoto, Japan). After precipitation of proteins at 4ºC by addition of four volumes of methanol, samples were chemically modified (derivatization in presence of 3-mercaptopropionic acid by ortho-phthalaldehyde and 9-fluorenylmethyl
chloroformate for primary and secondary amino acids, respectively), separated with a C18 column and detected by spectrophotometry at 338 and 262 nm. Fluxes $q_i$ (in mmol·gdw$^{-1}$·h$^{-1}$) were calculated as: $q_i = D \times (C_{i,\text{supernatant}} - C_{i,\text{medium}})/X_{\text{biomass}}$, where $C_i$ is the concentration of compound $i$ (mmol·L$^{-1}$), $X_{\text{biomass}}$ is the biomass concentration (gDW·L$^{-1}$), and $D$ is the dilution rate (h$^{-1}$).

**Enzyme activities: sampling, cell extract preparation and assay conditions**

An amount of cell culture containing 100 mg dry weight was centrifuged (4°C, 5 min, 8,000 rpm), washed once and resuspended in 3 to 6 mL 50 mM HEPES-KOH at pH 7.5, containing 15% glycerol supplemented with Halt Protease Inhibitor single-use cocktail, EDTA-free (Thermo Fischer Scientific, Rockford, IL). This suspension was divided into 0.5 mL aliquots added to 0.5 mg glass beads with 100 µm diameter (Bio-Spec Products, Bartlesville, OK) in screw capped tubes, snap-frozen in liquid nitrogen and stored at -20°C until further analysis. Frozen samples were thawed on ice and MgCl$_2$ was added to a final concentration of 2 mM. Cells were disrupted in a FastPrep FP120 homogenizer (BIO 101, Vista, CA) at a speed setting of 6, in 3 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 with a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific) using bovine serum albumin (BSA, 2 mg·mL$^{-1}$ stock solution; Pierce), containing 2 mM MgCl$_2$ 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 with the following differences: the coupling enzymes were not desalted, GAPDH was assayed with 5 mM arsenate and PGM was assayed in the absence of activator 2,3-bisphosphoglycerate. ALDH activity was not detected. All assays were checked for linearity and proportionality with increasing cell extract, with at least 4 technical replicates. 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.

**Total cell protein and total RNA**

Cells from each chemostat were taken and lysed with 2% SDS and incubated at 96°C for 2 h. The total amount of protein in the obtained cell lysates was determined by using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific) using BSA (2 mg·mL⁻¹ stock solution; Pierce) as the standard. Total protein data was obtained from technical triplicates and biological triplicates. TotRNA was acquired from cell lysate after cell disruption (Qiagen Tissue Lyser, 15 Hz, 2 cycles, 5 min each), extracted by phenol/ chloroform/ isoamylalcohol (25:24:1 v/v), and extracted again with chloroform/ isoamylalcohol (24:1 v/v). The totRNA was precipitated by the addition of isopropanol and by adding KAc to a final concentration of 150 mM, supplemented with Diethyl Phosphorocyanidate (DEPC). By vacuum-centrifugation the solvents were removed from the RNA. Finally, samples were mixed in MilliQ-DEPC until completely dissolved and measured at 260 nm by NanoDrop (Thermo Fisher Scientific Inc.). Data reported is an average of technical duplicates for each biological sample.

**DNA microarray analysis**

*L. lactis* cells (2 x 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 labeling were done as described previously. Labeled cDNAs were hybridized to full-genome DNA microarray slides of *L. lactis* MG1363, with the addition of probes for rProteins. All reagents and glassware for RNA work were treated with DEPC. RNA, cDNA quantity and quality, and the incorporation of the cyanine-labels were examined by NanoDrop (ThermoFisher Scientific Inc.) 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. 8). DNA microarray slide images were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Filtering of bad- and low-intensity spots and signals, data parsing, automated grid-based Lowess normalization, scaling, data visualization and outlier detection were performed using the Limma-package. We used the common reference design in which direct and indirect com-
**Figure 8.** Experimental setup. (A) Glucose limited chemostats of 1.5 L volume were fed with chemically defined medium for prolonged cultivation (CDMPC) with 25 mM glucose at a flow rate $F \text{mL}\cdot\text{h}^{-1}$. $C_{So}$ is the concentration of nutrients in the inflow, $C_{So^*}$ that in the chemostat and outflow, $X_{\text{biomass}}$ the biomass concentration inside the chemostat and outflow. (B) Various dilution rates were chosen to span the metabolic shift of *L. lactis*. (C) Chemostats were harvested after 10 volume changes for samples to determine culture density, mRNA and protein levels, and enzyme activities.
parisons were used to increase statistical significance. Fold changes are considered to be significantly altered when the $p$ value $\leq 0.05$.

**Proteomic analysis**

For protein expression profiling 2 x 250 mL of culture from each chemostat was collected by directly pouring it in pre-chilled centrifuge bottles containing chloramphenicol at a final concentration of 10 μg·mL⁻¹ (2.5 mL stock solution, 10 mg·mL⁻¹). The cells were harvested by centrifugation (4°C, 5 min, 8,000 rpm). 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 OD$_{600}$ of 50 in a total volume of 6 mL with 1 mM MgCl$_2$ were disrupted at 39 kPsi with a Constant Systems cell disrupter. 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 mL of 500 mM 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 labeling was performed three times (Fig. 8), according to the manufacturer’s protocol with few modifications as described elsewhere. The peptide mixture was subjected to chromatography and spectrometric analysis.

The pre-fractionation of peptides was performed on a silica based polysulfoethyl aspartamide strong cation exchange (SCX) column (catalogue number: 202SE0502 Poly LC inc., Columbia).
Proteomic data analysis and statistics
Raw proteome for each sample data consisting of four sets of 8-plex iTRAQ signal strengths annotated with a peptide and protein identifiers. Two data sets each originated from membrane and soluble protein fractions. Membrane and soluble protein fraction were analyzed 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 normalized by LOESS regression on an M-A transformation of the signals, as is common in microarray analysis. Using the assumption that the bulk of log-transformed signal ratios between different samples or between replicates will be ideally located symmetrically around 0 (no regulation) independently of the signal strength underlies this normalization technique. Since this technique is used originally when comparing only two samples, an adaptation for 8 samples was made. LOESS normalization was performed for each of the 28 unique pairs of samples within an 8-plex set, and these normalizations were reconciled by linear modeling. The normalized 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.

Fatty acid composition analysis
Samples from *L. lactis* chemostats, were pelleted and washed as described for proteomic analysis. All samples were transmethylated and analyzed on a gas chromatograph for acyl chain composition according to the methods described 82. The data presented is an average from the biological triplicates.

Constraint-based modelling: flux balance analysis and flux variability analysis
The genome scale metabolic model was based on that of *L. lactis* MG1363 (Flahaut, *et al.*, manuscript submitted) with modifications in growth and maintenance energy parameters which were estimated as described earlier 25. 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 dilution rate resulting in 12 models. 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 (FAME) 83.

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**Supplementary material Figures**

**Figure S1.** Quality control for proteomics data: For all the chemostats at different growth the cells were lysed and the soluble and membrane proteome was isolated as described in Materials and Methods. For each growth rate the relative quantification for proteins was performed on soluble and membrane fractions in independent experiments. This figure shows that the $2\log$ fold change regulation ratio of a protein in soluble or membrane fraction is correlated. Thus a change in the regulation ratio for a protein is equally well represented in both the fractions. The number of spectra obtained for a particular protein indicates its enrichment in either soluble or membrane fraction. Proteomics on exclusive membrane fractions allows investigating the minor changes in the abundance of membrane proteins with high confidence.
Figure S2. All amino acid consumption rates (q-rates) at various dilution rates.

Figure S3. rProtein ratios relative to the lowest growth rate 0.15 h⁻¹, for *L. lactis*, separated in early assembly, secondary assembly and late assembly.⁵⁸
**Supplementary material Tables**

**Table S1.** Transcriptome analysis of *L. lactis* MG1363 at varying growth rates. Tab1 (all) contains all genes sorted at accession number. Tab2 (regulon) contains the genes sorted based on their regulon and Tab3 (COG-cat) based on their COG-category. The table can be found on http://www.molgenrug.nl/supplementary_data/thesisEckhardt/Chapter3_TableS1.xlsx

**Table S2.** Proteome analysis of *L. lactis* MG1363 at varying growth rates. All measured proteins are sorted on their accession number. Indicated are the number of spectral reads and degrees of freedoms from the membrane (mem) and soluble (sol) fractions of every measured protein. The table can be found on http://www.molgenrug.nl/supplementary_data/thesisEckhardt/Chapter3_TableS2.xlsx

**Table S3.** List of (iso)enzymes of *L. lactis* MG1363 obtained from the proteome analysis. The table can be found on http://www.molgenrug.nl/supplementary_data/thesisEckhardt/Chapter3_TableS3.xlsx

**Table S4.** Correlations between transcripts and proteins of KEGG-categories (Tab1) and regulons (Tab2). The table can be found on http://www.molgenrug.nl/supplementary_data/thesisEckhardt/Chapter3_TableS4.xlsx