Lactococcus lactis systems biology

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Chapter 6

Summary and conclusion

This thesis describes a systems biology approach to explain the fermentative behavior of *Lactococcus lactis*, grown at specific varying growth rates. Different -omics technologies were accessible by collaboration between three research groups in The Netherlands. The coworkers of the Vrije Universiteit of Amsterdam provided the chemostat setup and performed the metabolomics experiments. Our team members of Membrane Enzymology at Rijksuniversiteit Groningen provided the expertise for proteomics, and our group Molecular Genetics at Rijksuniversiteit Groningen was responsible for performing genomics and transcriptomics studies and bioinformatics analyses. Technology Foundation Stichting Toegepaste Wetenschappen (STW) funded this project. Its aim was to stimulate the transfer of knowledge between technical sciences and industry. The fundamental knowledge derived from this research is integrated into a cellular model of *L. lactis* that will be able to predict fermentative behavior of this industrially very important microorganism. Our multi-omics data obtained from *L. lactis* growing at varying preset growth rates provides the fundament for this model. From an industrial perspective, such model is useful for further optimization of *L. lactis* as a microbial cell factory and for choosing the best growth conditions, while retaining desired properties. When cells of *L. lactis* were grown at varying growth rates in chemostats, under glucose limiting conditions, the bacteria employ a metabolic shift from mixed-acid to homolactic fermentation. *L. lactis* mainly produces formate, acetate and ethanol (mixed-acid) at low growth rates, while at high growth rates the major end-product of glycolysis is lactate (homolactic fermentation). Researchers have been trying to understand for a long time why *L. lactis* employs this metabolic shift [1–7]. The hypothesis for our study was that the shift from mixed-acid to homolactic fermentation is an outcome of evolutionary optimization of resource allocation, and is based on the predictions of the self-replicator model, which presumes that there is a tradeoff between investments in enzyme synthesis and metabolic yields for alternative catabolic pathways [8]. Most of the proteome in a lactococcal cell consists of glycolytic and ribosomal proteins. How much a cell invests in the different cellular 'modules' is defined as the protein investment and is, for an important part, determined by the transcriptional activity of its genes. A primary aim of our study was to characterize the protein investments in the different cellular modules at different growth rates (Fig. 1). In **Chapter 3**, a comprehensive and high-quality dataset of mRNA and protein ratios and enzyme activities of most of the glycolytic enzymes is presented. The data from our study shows that the transcription of the genes encoding glycolytic enzymes re-

mains equal with increasing growth rate. Also the protein levels and the kinetics of the glycolytic pathway enzymes hardly change when the growth rate changes. Yet, the metabolic flux data shows that under the growth rate-controlled conditions examined, a metabolic shift from mixed-acid to homolactic fermentation is observed. Thus, the theory of protein investment as a means to optimize the expressed proteins to the growth rate, does not explain the metabolic shift between mixed-acid and homolactic fermentation in *L. lactis*. The strain of *L. lactis* used in this study, MG1363, keeps a large majority of the glycolytic enzymes at a basal level to be prepared for possibly changing conditions.

The change in maximal catalytic capacity of a subset of glycolytic enzymes only partly explains how *L. lactis* shifts from mixed-acid to homolactic fermentation. In our experimental setup we determined enzyme kinetics *in vitro*. Even though the assay buffer mimicked the *in vivo* composition of the lactococcal cytoplasm, the kinetics of glycolytic enzymes should ideally be determined *in vivo*. Next to that, in order to point out what causes allosteric regulation, data of all allosteric modifications onto the glycolytic enzymes should be collected, as is recently attempted in the PHOSIDA database [9]. In our study we did not determine effectors, known to influence the metabolic shift, like the NADH/NAD^+ ratios [2], fructose-bisphosphate [10], or the phosphorylation state of HPr [11]. To increase completeness of the systems biology analysis, information of all intermediate metabolites should be determined. *In vivo* NMR is a useful technique for tracking glycolytic intermediates [6]. However, the relatively low sensitivity of *in vivo* NMR would require much thicker cell-suspensions than what we grew in chemostats. Alternatively, metabolites can be measured in cell extracts obtained using fast sampling and quenching methods.

Transcriptional changes in *L. lactis* under varying growth rates

In this thesis, a main focus was on the determination of the transcript level changes in cells of *L. lactis* when comparing four different growth rates. We present a method in **Chapter 2**, using the Limma package of R [12], which combines both direct and indirect comparisons of the transcriptomes at the different growth rates. This scheme improved the significance of all comparisons, thus enabling to detect very small changes in the transcriptomes. A thorough analysis of the transcriptome dataset revealed that mRNA levels of genes coding for the glycolytic enzymes hardly alter of cells growing at different growth rates in chemostat cultures.

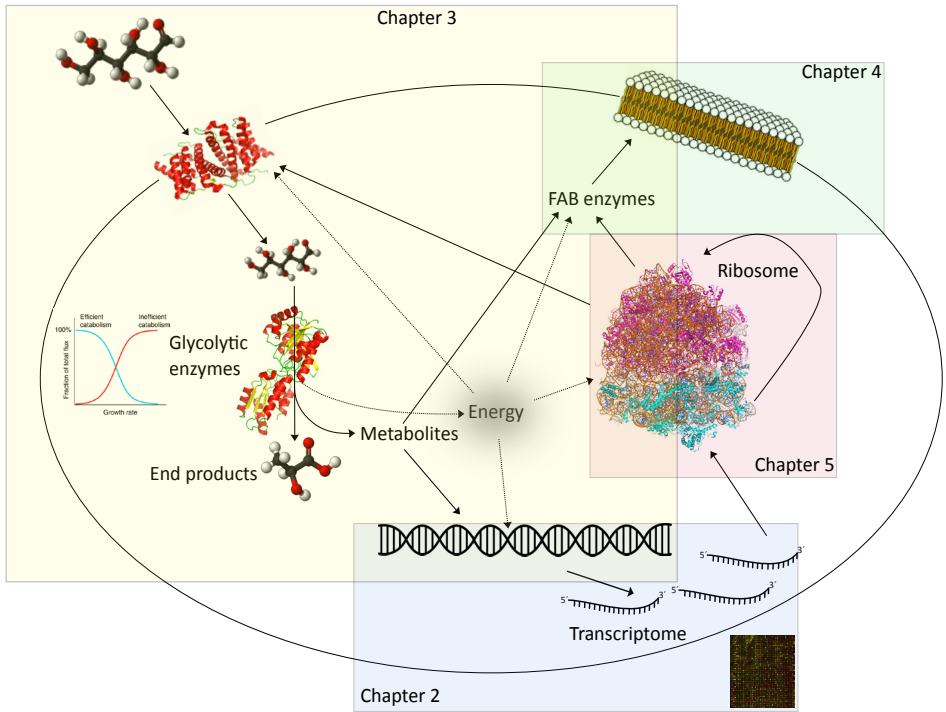

Figure 1. Subject of study of the various chapters in this thesis (coloured squares) projected onto the modular model of *L. lactis*. In Chapter 2, transcriptomics was performed, as part of the multi-omics study of *L. lactis* growing at different growth rates presented in Chapter 3. Regulation of fatty acid biosynthesis is detailed in Chapter 4. In Chapter 5, a characterization is given of YfiA, a protein essential for dimerization of ribosomes.

The transcriptional activities of genes of other important modules of the model were also characterized. Most of the genes encoding the ribosomal proteins show a very small increase with increasing growth rate. A non-linear correlation with the growth rate is seen for the transcription of the *arc*-genes, coding for the arginine catabolic ADI-pathway. Between growth rates of 0.15 h^{-1} and 0.5 h^{-1}, an increase in transcriptional activity of the arginine catabolism genes was followed by a steep decrease at 0.6 h^{-1}. Other important modules that were expected to be changed in the protein investment of the cell through changes in the transcriptional activity of the involved genes are fatty acid biosynthesis, membrane transport and cell division. We did not discern any general trends in the transcription of genes of those modules in response to an increase in the growth rate. All together we conclude that if the transcriptional activity of genes changes in response to growth rate, these changes are most often

very modest. The concentrations of proteins in the core proteome (e.g. those involved in metabolism, ribosomal proteins, etc.) are thereby less dependent on the change in transcript abundance.

Ribosomal composition at different growth rates

Ribosomes, throughout all kingdoms of life, are composed of two very important molecules: RNA and protein. As our data shows, the composition of a ribosome changes in response to the growth rate. Our experiments in which we determined the increase of totRNA/totProt and of rProteins hint that at increasing growth rates rRNA is less occupied by rProteins. Not all rProteins are functionally characterized, making it difficult to explain why some rProteins numbers per cell increase with the growth rate and why some others do not. The small subunit rProteins S1, S2, S5, S9, S19, S20, S21 and large subunit rProteins L7/L12, L10 and L12 do not respond to growth rate changes. None of the genes coding for these rProteins are located in *llmg2370-llmg2380*, the only cluster of genes that showed decreased transcription upon increasing growth rate. Many of the rProteins are not functionally characterized; they are thought to operate as a 'ribosomal glue'[13]. It is tempting to speculate that, (1) a subset of rProteins is minimally required for a ribosome to function as a ribozyme [14]. Another speculation may be that, (2) each ribosome has a different set of rProteins to stabilize it. Alternatively, (3) rRNA is synthesized to higher levels than needed at high growth rates. At lower growth rates, excessive rRNA is degraded and used as an energy source. At least the first hypothesis can be tested by isolating ribosomes from cells growing at different growth rates and examining the rRNA concentration in combination with quantitative MS/MS to reveal the identity and relative abundance of each ribosomal protein per ribosome.

The growth rate-adaptivity of rRNA and rProteins shows that at lower growth rates a minimal number of ribosomes per cell must be present. While at low growth rates, or after entering stationary phase, *E. coli* ribosomes are more prone to degradation by ribonucleases [15]. In **Chapter 5** we describe the functional properties of YfiA, a protein that prevents ribosomes from being degraded by ribonucleases. It does so by binding 70S ribosomes and dimerizing them. The research on *L. lactis* YfiA started by observing a significant decrease in *yfiA* transcription at high growth rates (Chapter 3, supplementary Table S1). The *yfiA* gene of *L. lactis* is annotated as a protein capable of binding both protein and RNA [16]. We concluded that, similar to YfiA of *E. coli*,

YfiA^{Ec} [17], *L. lactis* YfiA^{Ll} might play a role in ribosome binding. The *E. coli* proteins RMF and HPF stabilize the large and small ribosomal subunits and link two 30S subunits of two ribosomes together to form a ribosome dimer [18]. We show here that YfiA^{Ll} performs an opposite function in *L. lactis*: in contrast to YfiA^{Ec}, YfiA^{Ll} stimulates dimerization of ribosomes. YfiA^{Ll} contains a long C-terminal tail that is not present in YfiA^{Ec}. By performing a deletion analysis on YfiA^{Ll} we prove that this C-terminal extension is essential for ribosome dimer formation in *L. lactis*. All sequenced members of the family of Streptococcaceae contain YfiA homologs that are conserved in both their N-terminal domains, which have strong homology to the HPF protein of *E. coli*, and the extended C-terminal domains. The same organisms all lack proteins that resemble HPF and/or RMF of *E. coli*. In *Staphylococcus aureus*, the protein SaHPF is responsible for ribosome dimerization [20]. The amino acid sequences of SaHPF and YfiA^{Ll} are very similar; both contain the extended C-terminus (Chapter 5, Fig. 1). The results from our work and that of others suggests that bacteria can either dimerize ribosomes in two steps using HPF and RMF or in a single step employing a single protein that contains a C-terminal extension as is present in YfiA^{Ll} and SaHPF. In order to prove the interchangeability of the C-terminal domain of YfiA^{Ll} with RMF of *E. coli*, a complementation of YfiA$^{Ll1\text{-}126}$ with the latter protein should be made and tested for ribosome dimerization.

Fatty acid biosynthesis and regulation

Regulation of fatty acid biosynthesis (FAB) in *S. pneumoniae* and *E. feacalis* has been well studied and served as a basis for our studies [21–23]. In **Chapter 4**, a reconstruction is made of all enzymes required for FAB in *L. lactis*. Next to that, we show that FabT is a dedicated regulator for FAB in *L. lactis* and characterized the location of binding sites of FabT, both by bioinformatics techniques and with EMSA and DNAseI footprinting. When cultures of *L. lactis* are grown at different growth rates, the acyl chain composition of phospholipid membranes changes accordingly (Chapter 3, Fig. 6). How *L. lactis* alters the balance between saturated and unsaturated acyl chains in the membrane has not been solved yet. Dehydratase FabZ1 of *L. lactis* could function as an isomerase, like FabN in *E. faecalis* [21]. The substrate specificity of *E. faecalis* FabN is less constrained than that of dehydratase FabZ of *E. faecalis*, since the positioning of central helix α-3 is altered due to the specific placement of β3 and β4 sheets [21]. The crucial β3 and β4 sheets of *L. lactis* FabZ1 are mostly similar to those β-sheets of *E.*

faecalis FabN. By employing two FabZ dehydratase variants, one of which is capable of isomerizing acyl chains, *E. faecalis* can regulate the saturation of acyl chains in the phospholipid membrane [21]. Based on the synteny of the genes involved and the structural similarities between *L. lactis* FabZ1 and *E. faecalis* FabN and *L. lactis* FabZ2 and *E. faecalis* FabZ, it is likely that *L. lactis* regulates acyl chain saturation in the same way.

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