CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

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The work presented in this thesis focuses on the metabolism of glucose, galactose and lactose in *L. lactis*. In this concluding chapter, the obtained results will be summarized and future prospects will be discussed. The research performed was part of an EU-funded project called ‘Nutracells’, which aimed at proof the concept of gaining ‘healthy’ strains of lactic acid bacteria, useful for the dairy industry. The aim of the work presented in this thesis was to shift the preferences of *L. lactis* for the sugar glucose to lactose and/or galactose. The resulting end-products obtained by fermentation with these strains would contain less residual lactose and/or galactose, a favourable product characteristic for individuals suffering from lactose intolerance and/or galactosemia. Furthermore, by completely blocking glucose metabolism, glucose would be left over as an end-product and could, thus, function as a natural sweetener. Apart from the relevance of proving this concept for the industrial partners that are part of the nutracells programme, there is a major fundamental interest in the first steps of sugar metabolism and its regulation.

At the start of this work it was known that *L. lactis* could import glucose via PTS\textsuperscript{man\-glic}, encoded by *ptnABCD* and via (a) non-PTS transporter(s). To fully disable glucose metabolism, first the non-PTS route for glucose breakdown was blocked by deletion of *glk*, the gene encoding glucokinase. This enzyme catalyzes the phosphorylation of glucose to glucose-6-phosphate, which is the first step after non-PTS glucose transport. Subsequently, *ptnABCD* was disrupted in *L. lactis* NZ9000\Delta*glk* resulting in *L. lactis* NZ9000\Delta*glk*\Delta*ptnABCD*. Surprisingly, the latter strain could still use glucose as a substrate. Import of glucose had to be via a PTS, since the non-PTS-route was blocked by deletion of *glk*. Using DNA-microarrays, in which the expression of genes in the wildtype strain *L. lactis* NZ9000 was compared to that in *L. lactis* NZ9000\Delta*glk*\Delta*ptnABCD*, revealed several differentially expressed genes, of which genes of putative PTSs overexpressed in *L. lactis* NZ9000\Delta*glk*\Delta*ptnABCD* were, of course, most interesting as they potentially encode proteins involved in glucose transport. The genes *ptcB* and *ptcA* were five times more expressed in *L. lactis* NZ9000\Delta*glk*\Delta*ptnABCD* than in *L. lactis* NZ9000, which made them promising candidates. Interestingly, *ptcBA* together with *ptcC* were annotated as a cellobiose-transporting PTS (PTS\textsuperscript{cel}). Removing *glk*, *ptnABCD* and *ptcBA* in one strain resulted in a complete blockage of glucose metabolism, showing that the PTS\textsuperscript{cel} is actually a PTS\textsuperscript{cel/glic} (see Chapter 2). We confirmed that *ptcBAC* encodes a PTS transporting both glucose and cellobiose by growing *L. lactis* NZ9000, *L. lactis* NZ9000\Delta*ptcBA* and *L. lactis* NZ9000\Delta*glk*\Delta*ptnABCD*\Delta*ptcBA* (hereafter named *L. lactis* NZ9000Glc) in media containing galactose, glucose or cellobiose (data not shown). All three strains grew perfectly well on galactose. On
glucose, *L. lactis* NZ9000 and *L. lactis* NZ9000ΔptcBA grew, but *L. lactis* NZ9000Glc− did not. On cellobiose, only *L. lactis* NZ9000 grew happily, while both *L. lactis* NZ9000ΔptcBA and *L. lactis* NZ9000Glc− did not. These results further imply that cellobiose can only be transported via PTS_{cel/glc}.

The DNA-microarray technology was also employed to find the as yet unknown non-PTS glucose transporter(s), assuming that such transporters would be upregulated in *L. lactis* NZ9000ΔptnABCDΔptcBA, compared to strain NZ9000, when both strains would be growing on glucose. DNA-microarray results showed that the operon ytgBAH was highly upregulated in NZ9000ΔptnABCDΔptcBA (ytgA 11.3 times, ytgH 6.6 times, ytgB 2.9 times). Previous experiments had shown that ytgH (3.7 times) and ytgA (2.8 times) were also upregulated in NZ9000ΔglkΔptnABCD compared to NZ9000, both growing in the presence of glucose. The function of the proteins encoded by the small genes ytgBAH is as yet unknown, but since ytgB and ytgA both have 2 putative transmembrane domains and a high pI, they might be located in the membrane and involved in transport. To unravel whether ytgBAH indeed have a role in glucose uptake, a ytgBAH deletion strain in an *L. lactis* NZ9000 background as well as an NZ9000ΔptnABCDΔptcBA background, should be engineered and analyzed for differences in glucose transport. The same strategy could be followed for ymgH, which was overexpressed 3.2-fold in NZ9000ΔptnABCDΔptcBA compared to NZ90000, and also has 2 potential transmembrane domains and a high pI.

Once a glucose-negative strain of *L. lactis* was made that was unable to grow on glucose, the next step was to determine which lactose metabolic pathway is the most efficient to introduce lactose metabolic capacity in *L. lactis*. The first strategy was to introduce pMG820 (106), a plasmid containing the lac-PTS and tagatose-6-phosphate pathway in *L. lactis* NZ9000. *L. lactis* NZ9000[pMG820] grew well on medium supplemented with lactose. The second strategy was to introduce lacSZ from *Streptococcus thermophilus* ST11 in *L. lactis* NZ9000 with its normal cre-site and with a single mutation in cre, using the high copy number vector pIL253 (175). LacSZ-producing strains of *L. lactis* were able to grow in medium supplemented with lactose (whether or not the cre-box upstream of lacSZ was intact), but different clones did not show the same growth pattern. Sequencing data showed that the lacSZ constructs were correct. Possibly, this was caused by variations in the copy-number of the plasmid in the different clones, leading to different amounts of the membrane protein LacS. Unfortunately the same lacSZ-constructs cloned in the low-copy number vector pIL252 failed. The maximum growth rate of the strains
employing LacSZ for lactose metabolism was much lower than that of strains using lac-PTS together with the tagatose-6-phosphate pathway.

On the basis of the results obtained with pMG820 and lacSZ, we decided to focus on the PEP:PTS route for improving lactose metabolism in *L. lactis*. Plasmid pMG820 was introduced in *L. lactis* NZ9000 and *L. lactis* NZ9000Glc⁻ leading to, resp., *L. lactis* NZ9000Lac⁺ and *L. lactis* NZ9000Glc⁻Lac⁺. Since the latter strain can only use the galactose moiety of the disaccharide lactose for fermentation, it uses about twice as much lactose from the medium as *L. lactis* NZ9000Lac⁺ before growth ceases (due to the acidification of the medium). Besides the lower residual lactose concentration, also the relatively sweet glucose was produced as an end product by *L. lactis* NZ9000Glc⁻Lac⁺. Similar results were obtained in small-scale skim milk fermentations using this strain, which is promising for its application as an adjunct starter culture for industrial dairy applications.

During the process of making *L. lactis* NZ9000Glc⁻Lac⁺, several interesting strains were produced, which were investigated in more detail to study glucose import in *L. lactis*. Questions that still remained unanswered are: which glucose transporter is the most efficient for glucose import, and what effect does the use of a specific transport system have on the further (regulation of) metabolism of glucose? Transport assays showed that PTS\textsuperscript{mann/gl} is the major glucose transporter in *L. lactis*. These studies also revealed that removal of glk resulted in a lowered affinity of PTS\textsuperscript{mann/gl} for glucose, suggesting that the presence of glucokinase is important for maximal functioning of this PTS. A direct or indirect regulatory role for glucokinase was suggested. The enzymatic activity of Glk itself is regulated by metabolic components, other glycolytic enzymes or regulator proteins. The possible regulatory role of Glk in sugar metabolism still remains to be investigated along the following lines. *L. lactis* NZ9000Δglk should be complemented with a functional Glk, to examine whether the Glk-effect is diminished. Glk could also be overexpressed to see if the effect shown on the PTS\textsuperscript{mann/gl} by the glk-deletion can be abolished. An *L. lactis* strain has already been constructed in which glk can be overproduced by addition of nisin, NZ9000[pNZ8048-glk]. After induction with nisin, *L. lactis* NZ9000[pNZ8048-glk] functionally overexpressed Glk approximately 150 times compared to the strain that was not treated with nisin. An *in vivo* NMR experiment showed that the glucose fermentation pattern of *L. lactis* NZ9000[pNZ8048-glk] is comparable to that of *L. lactis* NZ9000; both have comparable glucose consumption rates and produce similar amounts of intracellular and extracellular metabolites. No further experiments have been performed with *L. lactis* NZ9000[pNZ8048-glk] so far.
It would be interesting to perform transport assays with NZ9000[pNZ8048-glk] and compare the results with previous experiments described in Chapter 3, to see if overexpression of Glk has a positive effect on the glucose transport efficiency of PTS\textsuperscript{\textit{man/vic}}. Another approach to show a link between Glk and PTS\textsuperscript{\textit{man/vic}} is to make a glk-GFP fusion protein and localize Glk-GFP in the cell. If Glk directly binds to PTS\textsuperscript{\textit{man/vic}}, it will be visible on the inside of the membrane instead of all throughout the cell, indicating a possible interaction of PTS\textsuperscript{\textit{man/vic}} with Glk. To find functional sites or amino acids in Glk, the effect of different mutations in the \textit{glk}-sequence, for example in the ROK-motif could be studied. To investigate if the regulation by Glk is direct or perhaps occurs via the global carbon metabolism regulator CcpA, protein-protein interaction studies could be performed. A more genetic approach would be to use a \textit{ccpA}-deletion strain of \textit{L. lactis} and a strain in which both \textit{glk} and \textit{ccpA} are deleted. An \textit{L. lactis} MG1363\textit{ΔccpA} deletion strain already present in our laboratory collection (225) was used to make an MG1363\textit{ΔglkΔccpA} double mutant. MG1363 is the parental strain of NZ9000 (MG1363\textit{pepN::nisRK}). Using DNA-microarrays, the transcriptomes of the different strains should be compared (NZ9000\textit{Δglk} to MG1363, MG1363\textit{ΔccpAΔglk} to MG1363 and MG1363\textit{ΔccpAΔglk} to NZ9000\textit{Δglk}) to unravel possible clues of the transcriptomes. A comparison of MG1363\textit{ΔccpA} and MG1363 has already been published (225).

Glucose metabolism of \textit{L. lactis} NZ9000, NZ9000\textit{Δglk}, MG1363\textit{ΔccpA} and MG1363\textit{ΔccpAΔglk} was also analyzed by \textit{in vivo} NMR. \textit{L. lactis} MG1363\textit{ΔccpAΔglk} showed a glucose fermentation pattern comparable to that of MG1363\textit{ΔccpA}. The glucose consumption rates and the formation of intracellular and extracellular metabolites of both strains were similar, but completely different from those of \textit{L. lactis} NZ9000 and NZ9000\textit{Δglk}. NZ9000\textit{Δglk} showed by far the most drastic change in glucose fermentation, thus it seems that the deletion of \textit{ccpA} overcomes the problems NZ9000\textit{Δglk} has.

Another interesting result during this work was the discovery of the anomeric specificities of the different glucose transport systems in \textit{L. lactis} NZ9000 (Chapter 3). PTS\textsuperscript{\textit{Cel/glc}} and the non-PTS glucose transporter(s) have a preference for \textit{β}-glucose, while PTS\textsuperscript{\textit{Glc/man}} displays no clear anomeric specificity and can use both the \textit{α}-anomer and the \textit{β}-anomer of glucose.

Having multiple systems for glucose transport, with different specificities for the two glucose anomers could improve survival under natural conditions when the cells need to compete with other microorganisms for glucose as sugar substrate. It would be interesting to gain insight in the exact mechanisms of transport of the different glucose transporters and to examine what determines the anomeric specificity.
All *L. lactis* glucose deletion strains described in Chapter 3 (NZ9000Δglk, NZ9000ΔptnABCD, NZ9000ΔptcBA and NZ9000ΔptnABCDΔptcBA) display a mixed acid fermentation pattern, while their parent strain *L. lactis* NZ9000 shows a homolactic fermentation mainly producing lactate. This shift to mixed acid fermentation is caused by key metabolic enzymes like pyruvate kinase (PK) and lactate dehydrogenase (LDH). The deletion strains had lower PK and LDH enzyme activities than *L. lactis* NZ9000. This is most probably not caused by transcriptional regulation of the genes for these enzymes, since they were not downregulated in a DNA-microarray experiment comparing the transcriptomes of *L. lactis* NZ9000ΔptnABCDΔptcBA and *L. lactis* NZ9000. The lower PK and LDH activities are caused by the concentrations of FBP and Pi in the cells (109, 153, 186, 191, 199), which are dependent on the rate of glucose metabolism, while the latter in turn depends highly on the glucose import system used. Lower activity of LDH results in a decreased lactate production, while production of other end products from pyruvate increases due to a higher activity of pyruvate formate lyase (PFL), shifting pyruvate metabolism to mixed acid fermentation. In addition to regulation of PFL at the enzyme level, the *pfl* gene was also transcriptionally activated in *L. lactis* NZ9000ΔptnABCDΔptcBA.

The genes encoding PTS<sup>cel/glc</sup> are located on the *L. lactis* chromosome in an operon together with *yecA* in the order *ptcB-ptcA-yecA-ptcC*. The *yecA* gene encodes a possible regulator protein. The PTS<sup>cel/glc</sup> deletion studies described in this thesis were all performed with a strain deleted in *ptcBA*. Since not the complete PTS<sup>cel/glc</sup> was disrupted, it would be interesting to engineer a *ptcC* deletion mutant and a *ptcBAPtcC* double deletion mutant, to see whether these strains display the same characteristics as NZ9000ΔptcBA. Furthermore, since *yecA* is in the middle of the PTS<sup>cel/glc</sup> operon, it would be interesting to study the function of YecA.

Chapter 4 describes the work performed on the improvement of galactose utilization by *L. lactis*. It was shown that *L. lactis* NZ9000 preferably uses the galactose non-PTS permease GalP followed by the Leloir pathway (GalMKT) for the metabolism of galactose. Surprisingly, a *galP* deletion strain still used galactose via the Leloir pathway, suggesting the presence of another non-PTS galactose permease. Deletion of *galPMK* resulted in total loss of the ability of the strain to metabolize galactose. To further investigate which other permease(s) transports galactose besides GalP, a DNA-microarray experiment should be performed in which the transcription profiles of *L. lactis* strains NZ9000 and NZ9000ΔgalP are compared, as
a gene encoding the putative additional galactose permease might be overexpressed in NZ9000ΔgalP to compensate for the loss of GalP. The lactose-PTS has been suggested to be able to use galactose as well as a substrate (92, 135), but so far this has not been proven. To analyze this possibility we introduced pMG820 (106), a 23.7 kb deletion derivative of pLP712, encoding PTS\textsuperscript{lac} and the tagatose-6P-pathway in \textit{L. lactis} NZ9000ΔgalPMK. The resulting strain indeed regained the capacity to use galactose as a substrate and produced tagatose-1,6-bisphosphate as an intermediate metabolite. Thus, this strain indeed uses the tagatose-6-phosphate pathway and not the Leloir pathway. This finding suggested that galactose was imported by the lactose-PTS encoded by \textit{lacFE}.

Recent sequencing studies discovered that no other sugar transporter is encoded by pMG820 (personal communication: U. Wegmann, Institute of Food Research, Norwich Research Park, Norwich, UK). Furthermore, we had to rule out the possibility that anoter galactose-PTS gene was encoded by the chromosome of \textit{L. lactis} MG1363, of which the activity only became visible when the tagatose-6-phosphate pathway genes (\textit{lacABCD}) were introduced on a plasmid (pMG820). Different combinations of \textit{lac}-genes were introduced in \textit{L. lactis} NZ9000ΔgalPMK by cloning them behind the nisin inducible promoter on pNZ8048, and galactose metabolic capacity was monitored after induction with nisin. The following preliminary results were obtained using \textit{L. lactis} NZ9000ΔgalPMK as a negative control and NZ9000pMG820 as a positive control. \textit{L. lactis} NZ9000ΔgalPMK[pNZ-lacFE] and NZ9000ΔgalPMK[pNZ-lacABCD] could not metabolize galactose, while NZ9000ΔgalPMK[pNZ-lacABCDFE] and NZ9000ΔgalPMK[pNZ-lacABCDFEG] were able to use galactose (A.R. Neves, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, unpublished results). These data suggest that \textit{lacFE} is necessary for galactose import and, thus, that LacFE is indeed a galactose/lactose-PTS. It also suggests that there is no other galactose-transporting PTS present on the \textit{L. lactis} NZ9000 chromosome. The introduction of the β-phosphogalactosidase encoded by \textit{lacG} is not required for the degradation of galactose. It would be interesting to introduce the plasmid with the nisin inducible \textit{lacABCDFEG} genes in \textit{L. lactis} NZ9000Glc\textsuperscript{−} discussed above, to examine whether the growth on lactose would improve when the lac-PTS and the tagatose-6P-pathway are overexpressed instead of normally expressed, when using pMG820.

In an effort to improve galactose metabolism in \textit{L. lactis} NZ9000 we tried to overexpress its favoured route of galactose degradation, the Leloir pathway (Chapter 4). Overexpression of \textit{galP(MKT)} led to accumulation of Gal1P and G1P, as shown by \textit{in vivo} NMR. Apparently, the bottleneck in galactose metabolism via
the Leloir pathway was at the level of α-phosphoglucomutase. The next step was to overexpress the gene encoding α-phosphoglucomutase alone and in combination with \textit{galP(MKT)}. Since a lactococcal gene for α-phosphoglucomutase turned out to be wrongly annotated in the \textit{L. lactis} genome (\textit{femD}), the \textit{pgmA} gene of \textit{S. thermophilus} was overexpressed in \textit{L. lactis} NZ9000. This indeed increased the α-phosphoglucomutase activity in the latter strain. Overexpression of \textit{S. thermophilus} \textit{pgmA} together with \textit{L. lactis galP(MKT)} relieved the bottleneck in galactose metabolism and improved the galactose consumption rate significantly. Apparently, the bottleneck in galactose metabolism is the reaction from glucose-1-phosphate to glucose-6-phosphate catalyzed by α-phosphoglucomutase. In this reaction UDP-glucose is produced, which is the glucosyl donor for the synthesis of glucose-containing polysaccharides. A next interesting step was to identify and characterize the α-phosphoglucomutase of \textit{L. lactis}.

In Chapter five of this thesis we described the identification of the genuine gene encoding \textit{L. lactis} α-phosphoglucomutase. Thus far, all α-PGMs described belong to the α-D-phosphohexomutase superfamily of proteins (171, 217). First, we overexpressed \textit{femD}, the only gene in the \textit{L. lactis} chromosome of which the product shows homology to known α-D-phosphohexomutases. FemD, however, did not display α-PGM activity. Purification of α-PGM activity from \textit{L. lactis} in combination with reverse genetics pinpointed the gene \textit{yfgH} as specifying α-PGM activity. Consequently, \textit{yfgH} was renamed \textit{pgmH}.

The α-PGM (\textit{pgmH}) of \textit{L. lactis} was characterized biochemically and the determination of the 3D-structure of this novel α-phosphoglucomutase is in progress. Functional analysis of \textit{L. lactis} α-phosphoglucomutase showed it to be the first characterized member of a novel α-PGM family. At the amino acid (aa) sequence level, \textit{L. lactis} α-phosphoglucomutase does not show any homology to the members of the α-D-phosphohexomutase superfamily. Instead, it has aa sequence homology with eukaryotic phosphomannomutases. Unlike the eukaryotic phosphomannomutases, which in general use both mannose-1-phosphate and glucose-1-phosphate, and bacterial α-PGMs belonging to the α-D-phosphohexomutase superfamily, which have a rather broad substrate preference, \textit{L. lactis} α-PGM shows strict specificity for α-G1P. In Chapter 5 (Fig. 8), \textit{L. lactis} α-PGM is placed in a phylogram with both characterized and putative phosphohexomutases from eukaryotic and bacterial sources.
Figure 1: Schematic overview of glucose, galactose, and lactose metabolism in L. lactis including all new metabolic components described in this thesis.

For details about the known glucose metabolic reactions see Chapter 3, Fig. 1. For details about the known galactose and lactose metabolic reactions see Chapter 1, Fig. 3. For details about the role of the histidine protein (HPr), see Chapter 1, Fig. 4. Besides donating phosphate to glucose via the PTSSs, HPr also delivers the phosphate group for uptake and phosphorylation of lactose and galactose imported by the PTSlacgal, but this detail is omitted from the figure for the sake of simplicity. All newly discovered metabolic proteins or their possible new roles in sugar metabolism are written in bold and/or encircled in dark-grey. PTScel/glc (encoded by ptcBAC) can transport and phosphorylate glucose and is specific for β-glucose. The PTSmanc/glc can use both α- and β-glucose. The non-PTS glucose transporter(s) is/are specific for β-glucose. Glucokinase (Glk), besides catalyzing the phosphorylation reaction of glucose, shows regulatory effects. PgmH was discovered to be the L. lactis α-phosphoglucomutase. Galactose can be transported by another non-PTS transporter apart from GalP, and it can be transported and phosphorylated by PTSlacgal encoded by lacFE.

Abbreviations: PTSman/glc, phosphoenolpyruvate-dependant phosphotransferase system (PEP:PTS) specific for mannose and glucose (encoded by ptnABCD); PTScel/glc, PEP:PTS specific for cellobiose and glucose (encoded by ptcBAC); PTSlacgal, PEP:PTS specific for lactose and galactose (encoded by lacFE); G6P, glucose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; TBP, tagatose-1,6-bisphosphate; Glk, glucokinase; PgmH, α-phosphoglucomutase; GalP, galactose permease; LacFE, EIIlac; PtnABCD, EIIglc/man; PtcBAC, EIIlac/gal; EI, Enzyme I; HPr, Histidine protein; HPr-His-P, HPr phosphorylated at histidine 15; HPr-Ser-P, HPr phosphoserylated at serine 46; CcpA, carbon catabolite protein A; +, activation; The letter P stands for a phosphate-group; ?, as yet unknown non-PTS glucose transporter; ??, newly discovered non-PTS galactose transporter(s) of which the gene(s) is/are as yet unknown.

L. lactis α-PGM is clustered with proteins with unknown function of the human-associated organisms Bifidobacterium longum and Propionibacterium acnes and with a protein with unknown function of the plant pathogen Gibberella zeae. Since L. lactis α-PGM is not homologous to α-PGMs of evolutionary closely related bacteria, we suggest that L. lactis might have obtained the gene for α-PGM by horizontal gene transfer. This might also be the case for the proteins with unknown function of the other organisms in the L. lactis α-PGM cluster. Of course it would be interesting to determine the function of these proteins with as yet unknown functions. L. lactis α-PGM showed around 25% identity with 2 human proteins. Since more and more genome sequences are becoming available, the near future might shed some more light on (the origin of) this intriguing new protein family.

L. lactis lacks an α-PGM of the α-D-phosphohexomutase superfamily, a property that seems to be unique among bacteria. The α-PGM encoded by pgmH was
demonstrated to be essential for *L. lactis*, which can be explained by the crucial role the enzyme fulfils in the physiology of the organism. α-PGM is not only required during galactose metabolism, but also during glucose metabolism, where its main function is to provide precursors for biosynthetic pathways e.g., for the production of exopolysaccharides. The UDP-Glc pool responds to variations in the level of α-PGM. Since *L. lactis* is an industrially relevant organism, the discovery of *L. lactis* α-PGM is interesting from the perspective of application. For example, exopolysaccharide production could be improved through manipulation of α-PGM (activity) in order to alter (improve) texture of food products or strains could be engineered with altered lytic capacity, which could lyse at a favourable time during the fermentation process.

Overall, the work described in this thesis yielded very interesting results at both the fundamental and more applied levels. All new metabolic components described in this thesis are depicted in Fig. 1. The applied goals of the Nutracells project were achieved. A strain with improved lactose metabolic capacity was produced by metabolic engineering. This strain produces glucose as end product, which can be used as a natural sweetener. Furthermore, galactose metabolism was improved once the metabolic bottleneck had been revealed and resolved. The next step for application would be to make all the strains food-grade and test these strains in industrial settings for the same traits. Application of the engineered strains is expected to be initiated in the USA rather than in Europe due to the more strict regulations and the lower consumer acceptance of GMO’s in the latter continent. With respect to the fundamental research objectives, this project gained a further and deeper insight in sugar uptake and degradation, mainly in the first steps of metabolism. The presence of an additional galactose non-PTS transporter in *L. lactis* was determined, the cellobiose-transporting PTS (*ptcBAC*) was shown to also have affinity for glucose, and the gene encoding a novel α-PGM activity was discovered and characterized. Although metabolic engineering strategies devised on the basis of theoretical know-how do not always lead to the expected results *in vivo*, along the way interesting fundamental metabolic insights are always gained.