39 The importance of balanced expression of glycolytic genes in *Lactococcus lactis*

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Introduction

Lactococcus lactis plays an important role in the manufacture of fermented dairy products. Usually, fermentation is performed in the presence of an excess of milk sugar and results in the formation of large amounts of lactic acid (homolactic fermentation) [1] and souring of the substrate in the production process of cheeses. Designing starter cultures genetically to acidify faster is of industrial interest, but also from a fundamental point of view, the question of what controls the flux through the glycolytic pathway is important.

In *L. lactis*, the genes (*pfk*, *pyk*, and *ldh*) [2] encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase and lactate dehydrogenase, are organized in one operon (*las*) and this organization is unique compared to other organisms. A transcriptional regulation of the *las* genes has been demonstrated [3], which increased the activity of the *las* enzymes 4-fold during the entry to stationary growth phase. Hence, the genetic organization may provide the cell with a way to regulate the glycolytic flux without considerable influence on the concentration of the glycolytic intermediates, by regulating enzyme activities early and late in the glycolytic pathway.

Here we look into the importance of the enzymes encoded in the *las* operon for *L. lactis* physiology. We constructed a series of mutants with altered expression of *pfk* or *ldh* and measured the corresponding changes in the glycolytic flux, growth rate and product formation, and we present experimentally determined flux control coefficients for LDH on the (i) glycolytic flux, (ii) lactate production and (iii) growth rate.

Materials and methods

The experimental procedures employed are described briefly here and will be described in more detail elsewhere [4]. *Lactococcus lactis* subsp. *cremoris* MG1363 was used as a model organism for modulating the expression of the *las* genes. Plasmids harbouring *pfk* and *ldh* [2] were kindly provided by A.J. Hillier. The upstream region of the *las* operon was obtained by inverse PCR. To modulate the *ldh* expression an integration vector pBF12α [5] was kindly provided by Dr. L. Brøndsted. The inactivation of *ldh* in MG1363 is described in [6]. The synthetic promoters have been described previously [7]. *L. lactis* strains were grown in batch culture (100 ml flasks) without aeration in either M17 or defined SA medium as described by [8]. The cultures were supplemented with either 1% of glucose, 1% fructose, 2% mannose, 2% maltose and incubated at 30°C. Growth was also performed in a BIOSTAT Q fermenter (BRAUN) as either batch cultures or as glucose limited chemostat cultures with a working volume of 600 ml SA or M17 supplemented with 0.25% glucose and the temperature was set at 30°C, stir at 200 rpm and pH was maintained at 7.0 by addition of 2M NaOH. The concentration of glucose and end products were analyzed by HPLC. The preparation of enzyme extracts and measurement of enzyme activities will be described elsewhere [4].

Results

Two-fold lowering of *pfk* expression has a high impact on the glycolytic flux

Plasmids were constructed in which the native promoter of the *las* operon was replaced which the synthetic promoters CP 25 and CP 29 [4]. By a double cross-over strain HWA 217 and HWA 232 were constructed (Fig. 39.1).

These mutants, HWA 217 and HWA 232 showed imbalance in the activities of phosphofructokinase, pyruvate kinase and lactate dehydrogenase (Table 39.1): PFK was lowered to 42 and 58%, respectively while PK and LDH remained close to the wild type level.

Surprisingly, the growth rate of HWA 217 and HWA 232 was lowered to 62% and 76%, respectively, and a similar decrease was seen for the glycolytic flux and for the rate of lactate production. These results suggest that PFK might have a high control on the growth rate and the glycolytic flux in this organism. In complex medium, M17, the growth rate of HWA 217 was decreased even further, to 50% of the wild type growth rate.

When sugar becomes limiting, e.g., in the chemostat, the metabolism of these bacteria is known to shift to mixed acid products (formate, acetate and ethanol) [1]. Therefore, if PFK limits the glycolytic flux in HWA 217 and HWA 232, we might see a similar shift in the product formation for these strains. However, the pattern

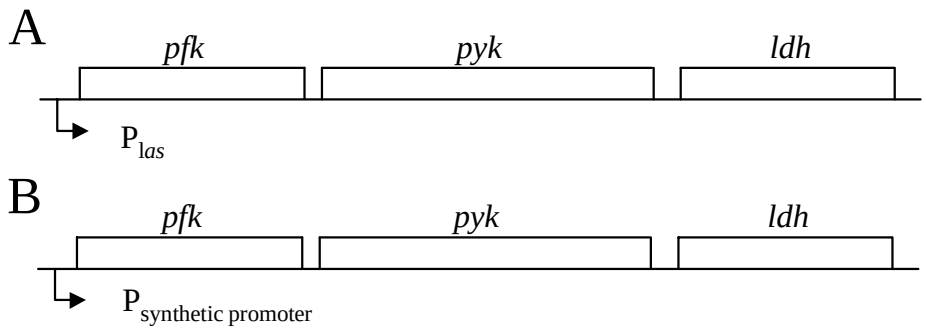

Fig. 39.1 Replacement of the promoter of the *las* operon with synthetic promoters (las mutants). (A) The *las* operon in the wild type strain MG1363 where the *las* promoter is transcribing *pfk*, *pyk* and *ldh*. (B) The *las* mutants in which the *las* promoter is replaced by synthetic promoters.

Table 39.1 Mutants with altered expression of the *las* operon (*las* mutants).

			Specific activity (% wild type)[c]		
Strains	Genotype[a]	Fermentation pattern[b]	PFK	PK	LDH
MG1363	wild type	homolactic	100 (14)	100 (6)	100 (3)
HWA217	CP25 – *las*	homolactic	42 (12)	115 (13)	137 (4)
HWA232	CP29 – *las*	homolactic	58 (15)	93 (10)	106 (12)

[a]The mutant strains are MG1363 derivatives. The synthetic promoters transcribing the *las* genes are shown.

[b]The pattern of fermentation was determined by HPLC. The c-mol recovery was calculated to 79–82%.

[c]The specific activity of PFK, PK and LDH in MG1363 is 26, 68 and 116 nmol/min·mg protein, respectively. The specific activity of the *las* enzymes in HWA 217 and HWA 232 is shown as the percentage of MG1363 and the standard deviation is shown in brackets. The values are the average of at least three independent measurements and within the experiments the activity from each strain was determined in triplicates.

of product formation was still mainly homolactic with only a slight increase in the concentration of the mixed acid products, formate and acetate, and other end products such as ethanol and acetoin could not be detected.

To investigate the cause of the altered physiology of strains HWA 217 and HWA 232, the strains were grown with different carbon sources (Table 39.2). In SA medium supplemented with glucose or mannose the specific growth rate of HWA 217 and HWA 232 was reduced which correlates well with the reduction of the glycolytic flux. By applying the substrate fructose, which circumvents the utilization of PFK (fructose is taken up as fructose-1-phosphate), the growth pattern of the mutant strains was similar to the wild type. Maltose is a substrate on which the growth is energy limited because the sugar is fed into glycolysis at a lower rate resulting in mixed acid formation.

Table 39.2 Growth of the *las* mutants on various substrates.

		Specific growth rate (h^{-1})[a]		
Substrate		MG1363	HWA217	HWA232
SA.IV	+glucose	0.74 (5)	0.46 (2)	0.56 (2)
	+mannose	0.73 (4)	0.46 (8)	0.55 (17)
	+fructose	0.70	0.73	0.72
	+maltose	0.39	0.36	0.40
M17	+glucose	1.14 (1)	0.57 (6)	–

[a]The specific growth rate, μ is the average of at least two independent measurements and the standard deviation in percentage is shown in brackets. The experiments where fructose and maltose were used as substrates were performed one time. The cultures were grown in batch.

With maltose, the strains had a low specific growth rate, but similar to the wild type, which shows that under these conditions, PFK is no longer limiting.

Although glycolysis is mostly a catabolic pathway, there is still a small flux routed from glycolysis into, e.g., nucleotides. A limitation in the reaction of PFK might have caused a lowering of the downstream glycolytic intermediates required in anabolic reactions. In rich medium the cells are provided with more building blocks than in defined medium. However, in rich medium M17 with glucose the mutants still showed a reduced growth rate as with defined medium, and we can therefore exclude a lack of building blocks as the cause.

By complementation with *pfk* on a plasmid the *las* mutants retained the specific growth rate as for MG1363. Together, these results clearly show that the low PFK activity was the direct cause of the disordered physiology of HWA217 and HWA232.

But it is still a puzzle why the fermentation pattern of the mutants remained homolactic. Another explanation for the phenotype could be that the low activity of PFK results in a toxic accumulation of sugar phosphates, which are toxic at elevated levels. If this was the case it might be possible to increase the specific growth rate by limiting the external concentration of glucose, as previously observed for a *Steptococcus mutans* LDH mutant [9]. We therefore performed a chemostat experiment where the growth rate of HWA 217 was gradually increased by slowly increasing the dilution rate. Indeed, the specific growth rate was enhanced to 1.11 h^{-1} in the chemostat, which was 95% higher than the rate obtained in the batch culture with excess of glucose present. This experiment shows that it is possible to obtain a higher growth rate (and presumably a higher glycolytic flux) by limiting the sugar supply to the cells! A likely explanation is that we thereby avoid a toxic accumulation of early intermediates such as glucose-6-phosphate.

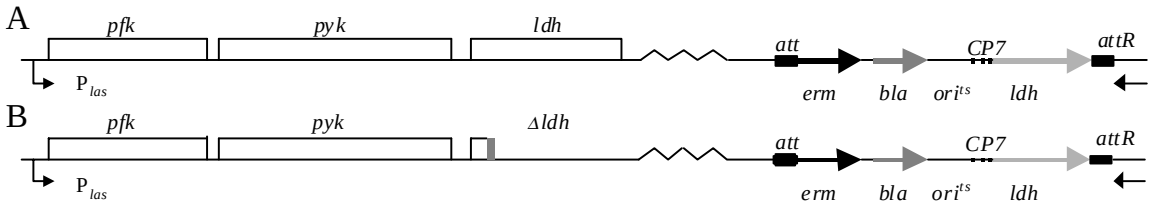

Fig. 39.2 Structures of mutants with altered expression of *ldh*. *ldh* mutants in which an additional *ldh* gene preceeded by the synthetic promoter CP7 is integrated in a phage (901) attachment site on the chromosome, in the wild type strain (A) or the null mutant (B) which is indicated by a gray box. The site-specific integration generates two sequences *attL* and *attR*, which is shown by black boxes.

Determination of flux control by lactate dehydrogenase

In order to determine the control exerted by LDH on the glycolytic flux a series of *ldh* mutants (Fig. 39.2) having activities of LDH from zero to 133% of the wild type were constructed [4]. These strains were grown as batch cultures in SA medium supplemented with glucose and samples were withdrawn for determination of the concentration of end products. The pattern of fermentation remained homolactic with decreasing LDH activity until a LDH activity of 58% was reached and then shifted to mixed acid when LDH activity was lowered to 11%.

To analyze the control of LDH on the (i) glycolytic flux, (ii) lactate production and (iii) growth rate, the control coefficients were estimated from the relative fluxes and enzyme activities. Control coefficients are only valid if the system is in a steady state or quasi-state, and we confirmed that the *ldh* gene was expressed at a constant level during growth in batch fermentation.

The control by LDH on the glycolytic flux was 0.06 at the wild type enzyme level when estimated from the function fitted to all the data points of the glycolytic flux. The flux control coefficient for the lactate production was estimated to −0.03. Finally, the control coefficient of the specific growth rate was estimated to 0.03 at the wild type level (Fig. 39.3).

Concluding remarks

We have shown that lowering the PFK activity to half of the wild type level dramatically influenced the glycolytic flux, the rate of lactate production and the specific growth rate. However, it was not possible from these data to estimate the control exerted by PFK on the glycolytic flux at the wild type enzyme level, due to a lack of mutants with PFK activities closer to and above the wild type level.

The growth rate of the PFK mutants in batch cultures of M17 medium was only half of the wild type growth rate, but increased almost to the wild type (batch)

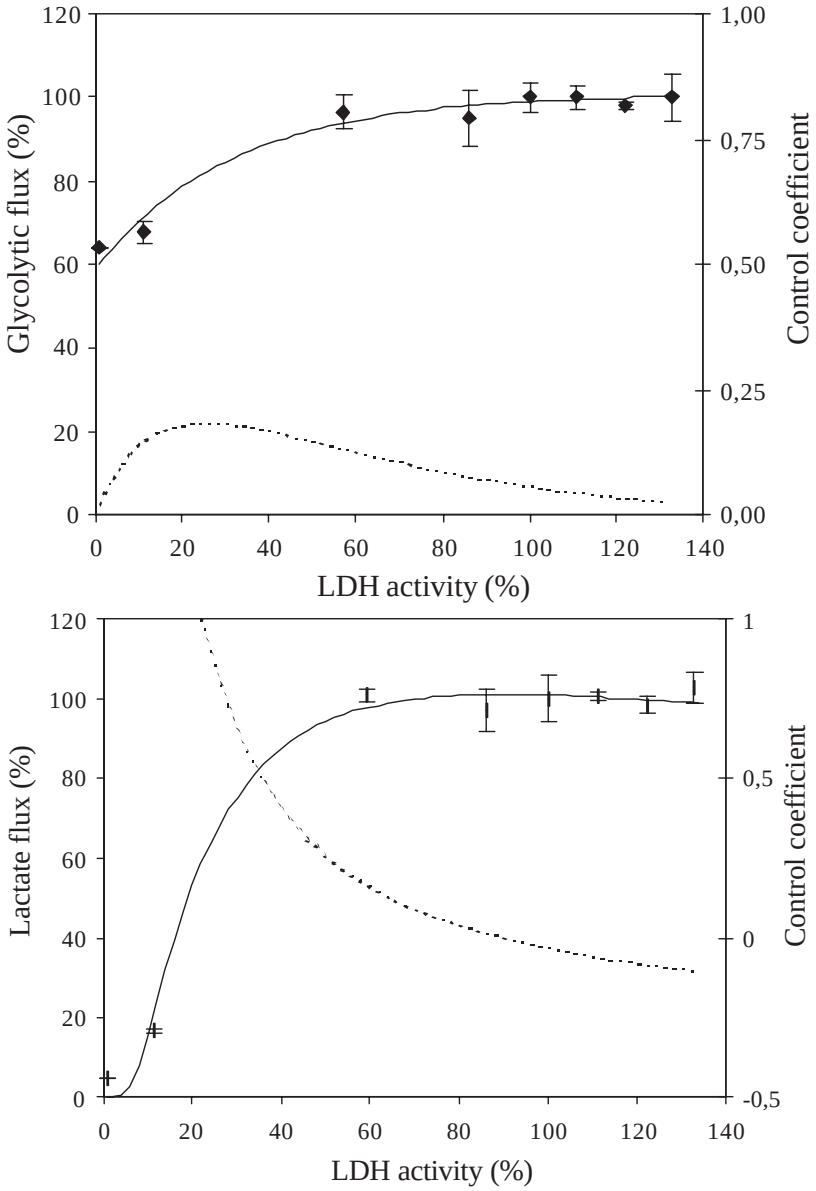

Fig. 39.3 Control exerted by LDH on the glycolytic flux and the lactate flux in *L. lactis.* The flux is shown as a function of the LDH activity of the *ldh* mutants (in % of the wild type flux) and indicated by diamonds. The flux data points are the average of at least two independent measurements and error bars indicate the standard derivation in percentage. The data point close to zero is from one experiment. All the glycolytic flux data points are fitted to a one phase exponential association function as shown with a solid line: $J_G(x) = a + b(1 - \exp(-cx))$. The values of a, b and c were: 58.95, 41.87, and 0.03153, respectively. The lactic flux data points are fitted to the following function as indicated by a solid line: $J_L(X) = ab^{(1/x)}x^c$. The values of a, b and c were: 590.4, $2.538 \cdot 10^{-13}$, and -0.3206, respectively. The control coefficients estimated from the curves are shown as broken lines.

growth rate when the cells were grown in a glucose limited chemostat. This result indicates that there is still an excess capacity of PFK activity in the mutant cells. An explanation for the low flux in the mutants could then be that the altered expression of PFK, relative to the other glycolytic enzymes in the system, results in an imbalance in the concentration of some intracellular metabolite(s) and subsequent down regulation, or even in toxic levels of such metabolites.

The control coefficients of LDH with respect to the glycolytic flux and the specific growth rate were determined to be close to zero at the wild type level. This result may be less surprising in view of the fact that these cells have an alternative route for conversion of pyruvate. What is perhaps more surprising is that the control exerted by LDH on the flux through the enzyme itself was also zero; apparently, these cells have homeostatic properties which allow them to maintain a constant flux to lactate, even with more than two fold change in LDH activity.

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