44 Is the glycolytic flux in *Lactococcus lactis* controlled by glycolysis itself?

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Introduction

Lactococcus lactis is used extensively in the manufacturing of fermented dairy products, where the primary function is the homofermentative conversion of sugar through glycolysis to the major end product lactate. The production of lactate is particularly important for the acidification of food products for preservation purposes. Thus, from an industrial point of view there is great interest in increasing the glycolytic flux and thereby the rate of acidification. Metabolic control theory shows that flux control can reside in any of the enzymes in the system, i.e., also in the numerous processes that consume the ATP generated in glycolysis. However, to investigate all the enzymes involved in the anabolic processes and quantify their contribution to flux control is unrealistic. A way to get around this problem is to introduce an additional ATP hydrolyzing enzyme, which does not interfere with other aspects of metabolism and thereby increase the ATP demand. We have previously shown how the glycolytic flux in *E. coli* can be increased by introducing uncoupled ATPase activity through the over expression of the F_1-subunits of the H^+-ATPase (Koebmann *et al.*, 1998; Koebmann *et al.*, manuscript in preparation). Thus, the glycolytic flux in this bacterium appeared to be controlled by the demand for ATP ($C^{J_{gluc}}_{demand} = 0.7$).

We recently cloned the *atp* operon from *L. lactis* MG1363, which enabled us to study the effect of over expression of F_1-subunits in *L. lactis* (Koebmann *et al.*, manuscript submitted). In this paper we show that when the F_1-subunits, α, β and γ are over expressed in *L. lactis* the growth rate decreased dramatically, and also the glycolytic flux decreased. This is in contrast to what was found for *E. coli.* Whether this is due to lack of control by demand for ATP, or if other secondary

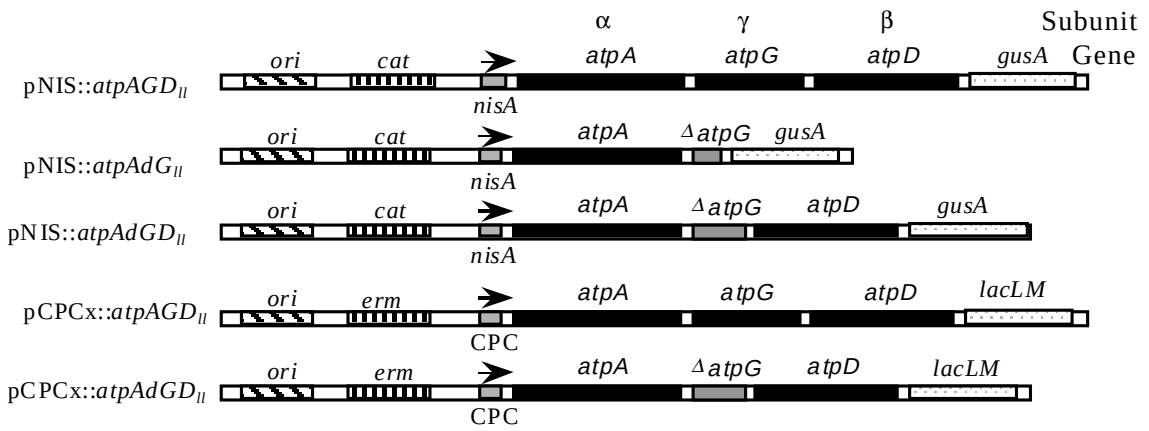

Fig. 44.1 Linear representation of the plasmids constructed for modulating ATPase activity in *L. lactis* (not drawn to scale). See text for further details. x: = different constitutive promoters (From Koebmann *et al.*, manuscript in preparation)

effects are involved is discussed.

Material and methods

Bacterial strains Cloning and plasmid propagation were performed using *E. coli* K-12 strain BOE270, derived from MC1000 (Casabadan *et al.*, 1980). The plasmid-free *Lactococcus lactis* subsp. *cremoris* MG1363 (Gasson, 1983) was used for studying the effects of uncoupled ATPase activity.

DNA techniques DNA isolation, cutting with restriction enzymes, treatment with T4 DNA ligase and Calf Intestine Phosphatase were carried out as described by Sambrook *et al.,* (1989).

Cloning of *atpAGD*$_{ll}$ and *atpAdGD*$_{ll}$ under the control of the inducible *nisA* promoter or artificial constitutive promoters Details of this work will be given elsewhere (Koebmann *et al.*, manuscript in preparation). Shortly, a fragment of *atpAGD*$_{ll}$ was obtained from the plasmid pMOS::*atpAGD*$_{ll}$, which carries a cloned PCR-fragment of *atpAGD*$_{ll}$ from *L. lactis* MG1363. The *atpAGD*$_{ll}$ fragment was cloned after the inducible *nisA* promoter in plasmid pNZ8010 (de Ruyter *et al.*, 1996), resulting in pNIS::*atpAGD*$_{ll}$ (Fig. 44.1).

The deletion of a part of *atpG* was made by cutting the plasmid pNIS::*atpAGD*$_{ll}$ and religating it by the use of two adaptors to reestablish the reading frame (pNIS::*atpAdG*$_{ll}$). A fragment from the original pNIS::*atpAGD*$_{ll}$ was subsequently cloned into pNIS::*atpAdG*$_{ll}$, resulting in pNIS::*atpAdGD*$_{ll}$ in which a part of *atpG* has been deleted (Fig. 44.1).

The pCPC library is a derivative of pAK80 containing a broad range of cloned artificial constitutive promoters upstream to the *lacLM* genes (Jensen and Hammer, 1998). The $atpAGD_{ll}$ fragment from pMOS::$atpAGD_{ll}$ or the $atpAdGD_{ll}$from pNIS::$atpAdGD_{ll}$were cloned in the pCPC library after the artificial promoters, resulting in several clones, which express the $atpAGD_{ll}$ or $atpAdGD_{ll}$genes to different extents (Fig. 44.1).

Growth experiments Growth experiments were performed with strains having $atpAGD_{ll}$ or $atpAdGD_{ll}$under control of either the inducible *nisA* promoter or a range of artificial constitutive promoters. Strains with the respective expression vectors were used as control. The strains were inoculated from overnight-cultures at 30°C into 90 ml SA-medium supplemented with 0.1% glucose and 5 μg/ml erythromycin (pCPC-vectors) or 5 μg/ml chloramphenicol (pNIS::$atpAGD_{ll}$). For the strain containing pNIS::$atpAGD_{ll}$ we also added different concentration of nisin. Regular measurements of $OD_{450/600}$ were made and HPLC samples taken for measuring the glycolytic flux.

Results and discussion

Expression of uncoupled ATPase activity has a negative effect on the glycolytic flux in *L. lactis*

We constructed plasmids that express the $atpAGD_{ll}$ genes of *L. lactis* from the inducible *nisA* promoter (Fig. 44.1). This combination of gene products, $\alpha_3\gamma\beta_3$, has in reconstitution experiments been found to be most active with respect to ATP hydrolysis *in vitro* (Dunn and Heppel, 1981), and *in vivo* experiments in *E. coli* confirmed this observation (Jensen, 1997). When the strain was streaked onto GM17 plates containing 5 μg/ml chloramphenicol and varying amounts of nisin (0–16 ng/ml), the induction of the F_1-subunits retarded growth. We also studied the effect of induction of ATPase activity in liquid cultures. *L. lactis* BK1128 containing pNIS::$atpAGD_{ll}$ was grown in defined medium (SA) supplemented with a limiting concentration of glucose, without nisin and in the presence of increasing concentrations of nisin. A strain containing only the vector pNZ8010, *L. lactis* BK1126, was grown in parallel as a reference. When nisin was added to the medium, the growth rate of the control strain decreased dramatically. This effect is probably related to the toxic nature of nisin itself. Therefore, the measurements on *L. lactis* BK1128 had to be compared to the control strain grown in the presence of the same nisin concentrations. The biomass yield on glucose decreased drastically with increased ATPase expression in the cells, which indicated that ATP was indeed being hydrolyzed. However, the growth rate was also significantly affected, see Table 44.1.

Table 44.1 Growth of *L. lactis* BK1128 (pNIS::$atpAGD_{ll}$) and *L. lactis* BK1126 (pNZ8010) in SA+0.15% glucose + 5γcam + (0,1,4) ng/ml nisin.

Nisin conc.	Plasmid	Specific β-gluc activity (MU)[a]	Biomass yield gdw/mmol glucose	Biomass yield %	Growth rate μ h^{-1}	Growth rate %
0 ng/ml	pNZ8010	2	0,0316	100	0.736	100
	pNIS::$atpAGD_{ll}$	3	0,0321	102	0.708	96
1 ng/ml	pNZ8010	2860	0,0256	100	0.349	100
	pNIS::$atpAGD_{ll}$	1593	0,0139	54	0.188	54
4 ng/ml	pNZ8010	4192	0,0237	100	0.217	100
	pNIS::$atpAGD_{ll}$	2179	0,0130	55	0.111	51

[a]The expressions of the *gusA* gene after $atpAGD_{ll}$ (Fig. 44.1) are given in the table as specific β-gluc activities (Miller units), and is an indication of the expression of the $atpAGD_{ll}$ genes.

Due to the effect of nisin on the control strain, we were unable to use these data to calculate the control exerted by the ATP demand on the glycolytic flux. The $atpAGD_{ll}$ genes were therefore cloned after a set of artificial constitutive promoters of different strengths and transformed into *L. lactis*. 98 individual clones with varying levels of expression of ATPase were obtained. Five strains were subsequently characterized with respect to the specific growth rate, μ, yield on glucose, Y_{gluc}, and glycolytic flux, J_{gluc}. These results are shown in Table 44.2. The expression of the F_1-ATPase subunits had a substantially negative effect on the growth rate as the expression level was increased. The effect on the growth yield was weaker, though the yield dropped to 68% of the initial value for the highest expression used.

The flux control coefficient of the demand for ATP in *L. lactis* appears to be negative

Less than 5% of the sugar taken up from the medium is used for cell mass. Consequently, the link between anabolism and catabolism is the ATP/ADP ratio and the proton gradient. This allows for an almost perfect splitting of the metabolism into a catabolic module (ATP supply) and an anabolic module (ATP demand). If we further assume that ATP consumption is strictly coupled to growth we can write:

$$\text{Substrate} \xrightarrow{e_1} \Delta G_p \xrightarrow{e_2} \text{Growth}$$

The flux control coefficients $C_{e_1}^{J_1}$ and $C_{e_2}^{J_1}$ of this simple scheme can be calculated from

$$C_{e_1}^{J_1} = \frac{\varepsilon_p^{e_2}}{\varepsilon_p^{e_2} - \varepsilon_p^{e_1}} \approx \frac{1}{1 - \frac{\Delta J_1 \%}{\Delta J_2 \%}} \tag{44.1}$$

Table 44.2 Expression of uncoupled F_1-ATPase activity (α, γ, β-subunits) in *L. lactis* (from Koebmann *et al.*, manuscript in preparation). Growth in SA medium + 0.1% glucose + 5 μg/ml erythromycin.

Strain	Plasmid	Specific β-gal activity (MU)[a]	Biomass yield gdw/mmol glucose	Growth rate μ h^{-1}	Glucose flux mmol glucose /h/gdw	Biomass yield %	Growth rate %	Glucose flux %
BK1010	pAK80	0	0.0323	0.741	22.9	100	100	100
BK1546	pCPC69::*atpAGD*$_{ll}$	0.17	0.0324	0.760	23.5	100	103	102
BK1540	pCPC63::*atpAGD*$_{ll}$	5.88	0.0332	0.726	21.9	103	98	95
BK1552	pCPC75::*atpAGD*$_{ll}$	179	0.0284	0.567	19.9	88	77	87
BK1505	pCPC6::*atpAGD*$_{ll}$	362	0.0233	0.441	18.9	72	59	82
BK1517	pCPC33::*atpAGD*$_{ll}$	488	0.0218	0.363	16.6	68	49	73

[a]The expressions of the *lacLM* genes after *atpAGD*$_{ll}$ are given in the table as specific β-gal activities (Miller units), and is an indication of the expression of the *atpAGD*$_{ll}$ genes.

$$C_{e_2}^{J_1} \approx \frac{1}{1 - \frac{\Delta J_2 \%}{\Delta J_1 \%}} \tag{44.2}$$

if we assume small perturbations with uncoupled ATPase (Westerhoff and van Dam, 1987; Koebmann *et al.*, 1998).

When we use eqs. 44.1 and 44.2 to calculate the control by supply and demand on the glycolytic flux for each level of ATPase activity, the control by ATP supply is greater than 2 and the control by ATP demand is found to be negative. Clearly, these data indicates that something is completely wrong, since negative flux control is impossible in a linear pathway. HPLC data from the experiment in Table 44.1 showed that the clones were all still homolactic, and did not shift to mixed acid production; in theory such a shift would provide the cell with an extra ATP per glucose. Intuitively we might therefore have expected this shift to take place, and the absence of mixed acid products could indicate that an explanation on the decreased flux may lie elsewhere, for instance that the expression of F_1-subunits interferes with other aspects of Lactococcal metabolism.

Does the over expression of F_1 complexes interfere with the normal proton pumping H^+-ATPase complexes?

The structure of the F_1F_0-ATPase complex consists of two parts; a membrane integral part, F_0, which forms a proton channel, and a soluble part, F_1, which contains the catalytic site for ATP hydrolysis. In *L. lactis* the role of the H^+-ATPase is to maintain the proton gradient across the cytoplasmic membrane. To test how important the H^+-ATPase is for growth of *L. lactis*, we replaced the chromosomal *atp*

promoter with an inducible *nisA* promoter (Koebmann *et al.*, manuscript submitted). When grown at 30°C on GSA/GM17 plates (buffered at pH 7) with different concentrations of nisin the growth of the strain decreased dramatically with decreasing concentration of nisin, and was completely abolished in the absence of nisin. A negative effect on the growth was also observed at high nisin concentrations. However, this effect is probable caused by the toxicity of nisin itself, since a reference strain showed similar effects. This result shows that the H^+-ATPase is essential for growth of *L. lactis* under these conditions, presumably because it is essential for maintaining the cytoplasmic pH at an acceptable level.

The H^+-ATPase consists of eight subunits, which are all essential for the function of the F_1F_0-ATPase complex. Thus, a reduction in the level of any subunits from the complex will result in a lower activity of the H^+-ATPase, which may eventually lead to a lower growth rate. Many of the ATPase subunits have affinity for each other. According to the literature there appear to be interactions between a part of the γ-subunit and the ε-subunit (Dunn, 1982). Also, interactions between the α-subunit and δ-subunit seem to exist (Ogilvie *et al.*, 1997). When over expression of the F_1-subunits α, β and γ is engineered, there is a chance that some of the subunits from the H^+-ATPase will be titrated, resulting in non-functional H^+-ATPase complexes. To test this hypothesis with respect to the ε-subunit we constructed a clone where the internal part of the gene coding for the γ-subunit, *atpG*, was deleted and α and β were still intact (*atpAdGD*$_{ll}$). The deleted region appears to code for the part of γ that interacts with ε (Watts *et al.*, 1996; Tang *et al.*, 1996). The genes were cloned after a set of constitutive promoters (Fig. 44.1), and tested for growth in defined medium with a limited amount of glucose. However, also these clones affected the growth rate of *L. lactis*, indicating that titration of the ε-subunit might not be the (sole) problem here (data not shown).

Concluding remark

From the experiments in this paper we cannot solve the issue of what controls glycolysis in *L. lactis*. The data could indicate either that (i) control resides in glycolysis itself (or glucose uptake), or (ii) that perturbation of the membrane bound H^+-ATPase or some vital anabolic functions takes place, which then results in inhibition of cell growth in the presence of the uncoupled ATPase. These two options would amount to a catabolic and an anabolic limitation respectively. In order to discriminate between the two, we are currently measuring the intracellular concentrations of ATP and ADP. If the ATP/ADP ratio is lower in the cells containing the uncoupled ATPase, then it indicates control in glycolysis; If the ATP/ADP ratio is higher than in the normal cell, then it indicates that anabolism is disturbed by other means than a low energy state. We are also looking more direct into whether the over expression of F_1 subunits affects the activity of membrane bound H^+-ATPase, in inverted membrane vesicles from these cells.

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References

Casabadan, M.J. and Cohen, S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **138**, 179–207.

de Ruyter, P.G., Kuipers, O.P. and de Vos, W. M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin *Appl. Environ. Microbiol.* **62,** 3662–3667.

Dunn, S.D. (1982) The isolated γ subunit of *Escherichia coli* F_1 ATPase binds the ε subunit. *Biol. Chem.* **257**, 7354–7359.

Dunn, S.D. and Heppel, L.A. (1981) Properties and functions of the subunits of the *Escherichia coli* coupling factor ATPase. *Arch. Biochem. Biophys.* **210**, 421–436.

Gasson, M.J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**, 1–9.

Jensen, P.R. (1997) International patent application number PCT/DK97/00373, publication number WO 98/10089.

Jensen, P.R. and Hammer, K. (1998) The Sequence between the consensus sequences modulates the strength of prokaryotic promoters. *Appl. Environ. Microbiol.* **64**, 82–87.

Koebmann, B.J., Nilsson, D., Snoep, J.L., Westerhoff, H.V. and Jensen, P.R. (1998) The glycolytic flux in *E. coli* appears to be controlled by the demand for ATP, pp. 205–210. In C. Larsson, I.-L. Påhlman, and L. Gustafsson (eds.), BioThermoKinetics In The Post Genomic Era. Chalmers Reproservice, Göteborg, 205–210.

Ogilvie, I., Aggeler, R. and Capaldi, R. A. (1997) Cross-linking of the δ subunit to one of the three α subunits has no effect on functioning, as expected if δ is a part of the stator that links the F_1 and F_0 parts of the *Escherichia coli* ATP synthase. *J. Biol. Chem.* **272**, 16652–16656.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2^{nd} ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Tang, C. and Capaldi, R.A. (1996) Characterization of the interface between γ and ε subunits of *Escherichia coli* F_1-ATPase. *J. Biol. Chem.* **271**, 3018–3024.

Watts, S.D., Tang, C. and Capaldi, R.A. (1996) The stalk region of the *Escherichia coli* ATP synthase *J. Biol. Chem.* **271**, 28341–28347.

Westerhoff, H.V. and van Dam, K (1987). *Thermodynamics and Control of Biological Free Energy Transduction*. Elsevier, Amsterdam.