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Effect of pyruvate kinase overproduction on glucose metabolism of *Lactococcus lactis*

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Lactococcus lactis strain NZ9000(pNZ*pyk*), which overproduces pyruvate kinase (PK), was constructed. The pNZ*pyk* plasmid carries the P_{nisA}–*pyk* transcriptional fusion, and the overexpression of its *pyk* gene was accomplished by using the nisin-inducible expression system of the NZ9000 strain. *In vivo* ^{13}C- and ^{31}P-NMR spectroscopy was used to evaluate the effect of this modification on the metabolism of glucose in non-growing cells. A detailed description of the kinetics of glucose, end products, glycolytic intermediates, NAD^+ and NADH was obtained. A 15-fold increase in the level of PK did not increase the overall glycolytic flux, which, on the contrary, was slightly reduced. Significant differences were observed in (i) the level of 3-phosphoglycerate (3-PGA) and phosphoenolpyruvate (PEP), metabolites associated with starvation; (ii) the rate of fructose 1,6-bisphosphate (FBP) depletion upon glucose exhaustion; and (iii) the NAD^+/NADH ratio during glucose catabolism. In the mutant, the rate of FBP consumption after glucose depletion was notably accelerated under anaerobic conditions, whereas 3-PGA and PEP decreased to undetectable levels. Furthermore, the level of NAD^+ decreased steadily during the utilization of glucose, probably due to the unanticipated reduction in the lactate dehydrogenase activity in comparison with the control strain, NZ9000(pNZ8020). The results show that PK is an important bottleneck to carbon flux only when glucose becomes limiting; in the overproducer this constriction was no longer present, as evidenced by the faster FBP consumption and lack of accumulation of 3-PGA and PEP in anaerobic as well as aerobic conditions. Despite these clear changes, the PK-overproducing strain showed typical homolactic metabolism under anaerobic conditions, as did the strain harbouring the vector plasmid without the *pyk* insert. However, under an oxygen atmosphere, there was increased channelling of carbon to the production of acetate and acetoin, to the detriment of lactate production.

INTRODUCTION

Lactic acid bacteria are suitable model systems for studying the mechanisms involved in the regulation of sugar transport and metabolism. In particular, the relative metabolic simplicity of *Lactococcus lactis* makes it an ideal candidate for these studies. Regrettably, in spite of the wealth of metabolic information and ingenious genetic tools available for *L. lactis*, we are still far from having a comprehensive understanding of sugar metabolism and regulatory pathways in this model organism. The accomplishment of this goal has been hampered by the complexity of global metabolism, which consists of multiple interlocked pathways connected via common metabolites and cofactors through various degrees of genetic and metabolic regulation (Bailey, 2001). Molecular biology has been useful in dissecting complex systems into parts that are easier to analyse and understand; however, for obtaining a deep insight into metabolic regulation, a comprehensive knowledge of the cells as a whole is required. This achievement will be essential for the rational design of reliable engineering strategies to reroute carbon fluxes to the production of desired products.

Glycolytic regulation in *L. lactis* has been related to the high fructose 1,6-bisphosphate (FBP) concentration that activates lactate dehydrogenase (LDH) and pyruvate kinase (PK), directing the flux towards the production of lactate. Accordingly, accumulation of phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3-PGA) in starved cells is regarded

Abbreviations: FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); KP_i, potassium phosphate; LDH, L-lactate dehydrogenase (EC 1.1.1.27); P_i, inorganic phosphate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PK, pyruvate kinase (EC 2.7.1.40); 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.

as a consequence of PK inhibition by high inorganic phosphate (P_i) and low FBP concentrations associated with sugar depletion (Mason *et al.*, 1981; Thompson, 1987). Recent studies have questioned the role of FBP and postulated that inhibition or activation exerted by the cellular NADH/NAD^+ ratio on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or LDH is the main issue regulating glycolysis and the shift from homo- to heterolactic fermentation (Garrigues *et al.*, 1997). On the other hand, based on non-invasive NMR measurements of NAD^+ and NADH, we have shown that the glycolytic flux in resting cells is not appreciably influenced by the NADH/NAD^+ ratio (Neves *et al.*, 2002b). Recently, using an approach that involves manipulation of enzyme activities in small steps above and below the wild-type level and metabolic control analysis, Jensen and collaborators have shown that GAPDH has no control over glycolytic flux (Solem *et al.*, 2003). Similarly, LDH and also phosphofructokinase (PFK) have been ruled out as major sites of control of lactate production in *L. lactis* (Andersen *et al.*, 2001b; Koebmann *et al.*, 2002b). Hence, the control of glycolysis in *L. lactis* remains elusive.

PK (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis, where the phosphoryl group of PEP is transferred to ADP to form pyruvate and ATP. The reaction is irreversible under physiological conditions and has long been considered as critical for the regulation of metabolic flux in the second part of glycolysis. This central position is evident from the regulatory properties of PK, a typical allosteric protein (Valentini *et al.*, 2000). The lactococcal enzyme has been purified and characterized (Crow & Pritchard, 1976, 1982). It is activated by FBP, the activity being a sigmoidal function of the activator concentration, and the affinity for PEP and ADP is also enhanced by FBP. Furthermore, the K_a for FBP increases drastically in the presence of low concentrations of P_i. The magnitude of the FBP and P_i pools in *L. lactis* has been assessed in several studies; FBP accumulates up to 50 mM in the presence of glucose or lactose, whereas high concentrations of P_i, 3-PGA, 2-PGA and PEP are typical of starved cells (Thompson & Thomas, 1977; Thompson & Torchia, 1984; Neves *et al.*, 1999, 2002b).

Early studies provided evidence for a pivotal regulatory role of PK in the glycolytic metabolism of *L. lactis* (Mason *et al.*, 1981; Thompson & Torchia, 1984). These data prompted us to evaluate the effect of PK overproduction on the physiology of this model organism and to investigate the role of this enzyme in the regulation of the overall glycolytic flux. NMR techniques were used to monitor the kinetics of the intracellular pools of several glycolytic metabolites, NAD^+, NADH, ATP and P_i in living cells of *L. lactis* overproducing PK.

METHODS

Materials. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, FBP, PEP, LDH and NADH were purchased from Roche Molecular Biochemicals. DyNAzyme DNA polymerase was from Finnzymes. Oligonucleotides were synthesized by MWG-Biotech. [1-^{13}C]Glucose (99 % isotopic enrichment) and [6-^{13}C]glucose (99 %) were supplied by Campro Scientific. Other chemicals were reagent grade and obtained from Sigma or Aldrich.

Bacterial strains and media. *Escherichia coli* MC1061 was grown in Luria–Bertani broth with aeration at 37 °C. *Lactococcus lactis* strains MG1363 (Gasson, 1983), NZ9000 (expressing the *nisRK* regulatory genes; Kuipers *et al.*, 1998) and its derivatives were routinely cultivated without aeration in M17 medium supplemented with 1 % (w/v) glucose. Chloramphenicol was used at a concentration of 20 or 5 μg ml^{-1} for selection of pNZ8020-based plasmids (de Ruyter *et al.*, 1996) in *E. coli* or *L. lactis*, respectively. Bacterial growth was monitored spectrophotometrically at 600 nm.

DNA techniques and transformation. All manipulations with recombinant DNA in *E. coli* MC1061 (used as an intermediate host for cloning) were carried out according to standard procedures (Sambrook *et al.*, 1989) and as specified by the enzyme manufacturers. Chromosomal and plasmid DNA of *L. lactis* was isolated as described by Leenhouts *et al.* (1989) and O'Sullivan & Klaenhammer (1993), respectively. PCR reactions were performed in a total volume of 100 μl with 10 mM Tris/HCl (pH 8·8), 2 mM $MgCl_2$, 50 mM NaCl, 200 μM each deoxyribonucleoside triphosphate, 1 U DyNAzyme, 50 pmol of each primer and 1 μg template DNA obtained from MG1363. Amplification was performed in 25 cycles, each cycle consisting of a denaturation step at 95 °C for 1 min, a primer annealing step at 55 °C for 1 min, and a primer extension step at 72 °C for 2 min. Preparation of competent *L. lactis* cells and electrotransformation was performed as described by Dornan & Collins (1990).

Construction of plasmids and mutant strains. Primers Pyk1 (5′-GCGCTGGATCCCGCATAATTAGGGG-3′) and Pyk2 (5′-CGTATGAATTCGCCTGTCGATAGCGG-3′), based on the sequence of the PK gene (accession no. LO7920) published previously (Llanos *et al.*, 1993), were designed to introduce *Bam*HI and *Eco*RI restriction sites (underlined in the primer sequences) upstream and downstream, respectively, of the *pyk* gene coding region. A PCR product with the expected size (1·6 kb) was obtained and cloned as a *Bam*HI–*Eco*RI fragment into the similarly digested, high-copy-number shuttle vector pNZ8020 under control of the *nisA* promoter. The ligation mixture was transformed into *E. coli* MC1061, and a recombinant plasmid carrying the appropriate insert (designated pNZ*pyk*) was selected. The pNZ*pyk* and pNZ8020 plasmids were independently introduced by electroporation to *L. lactis* NZ9000 for further studies.

Fermentation under controlled conditions. *L. lactis* cultures of NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) were grown at 30 °C in a 2 litre fermenter in the defined medium (CDM) described by Poolman & Konings (1988), containing 1 % (w/v) glucose, 5 μg chloramphenicol ml^{-1} and 1 ng nisin ml^{-1}. Strain NZ9000 was grown in the same medium without addition of chloramphenicol and nisin. For the measurements of pyridine nucleotide pools, [5-^{13}C]nicotinic acid (5 mg l^{-1}), synthesized as described before (Neves *et al.*, 2002a), was supplied, and aspartate and asparagine were omitted. The pH was kept at 6·5 by automatic addition of 10 M NaOH and a low agitation rate (70 r.p.m.) was used to keep the fermentation broth homogeneous. Anaerobic conditions were established by gassing the medium with argon for 2 h before inoculation (4 % inoculum with a culture grown overnight in the same medium); for aerobic growth, the dissolved oxygen was monitored with an oxygen electrode (Ingold). A specific air tension of 90 % was maintained by automatic control of the airflow. Growth was evaluated by measuring the turbidity of the samples at 600 nm (OD_{600}) and calibration against cell dry weight measurements.

Preparation of ethanol extracts for analysis by ^{31}P-NMR and quantification of phosphorylated metabolites. Ethanol extracts were prepared from culture samples (approx. 70 mg protein for each

extract) obtained during growth as previously described (Ramos *et al.*, 2001), and the concentration of intracellular metabolites was calculated from the peak areas in ^{31}P spectra by comparing with the area of the resonance due to methylphosphonic acid added as internal standard. Assignment of resonances was achieved by addition of the pure compounds to the extracts or by comparison with previous studies (Ramos & Santos, 1996; Ramos *et al.*, 2001).

In vivo* NMR experiments.** Cells were harvested in the mid-exponential phase (OD_{600} 2·2), centrifuged and suspended in 50 mM potassium phosphate (KP_i) buffer, pH 6·5, to a protein concentration of approximately 13 mg ml^{-1}. *In vivo* NMR experiments were performed under anaerobic (argon atmosphere) or aerobic conditions (oxygen saturation) as described previously (Neves *et al.*, 1999). [1-^{13}C]Glucose (or [6-^{13}C]glucose) was added and the time-course for its consumption, product formation, and build-up and consumption of intracellular metabolite pools was monitored. After glucose exhaustion, and when no changes in the resonances due to end products and intracellular metabolites were observed, a cell extract was prepared (Neves *et al.*, 1999): briefly, an aliquot of the cell suspension was passed through a French press (twice at 120 MPa); the resulting extract was incubated at 80 °C for 15 min in a sealed tube, cooled on ice; the cell debris was removed by centrifugation (30 000 ***g, 10 min, 4 °C) and the supernatant (denoted hereafter as total NMR-sample extract) was stored at −20 °C.

An aliquot of the cell suspension used for *in vivo* NMR was saved to prepare a crude extract and measure the level of PK overproduction. Protein concentration in cell suspensions was determined by the Lowry method using bovine serum albumin as a standard after disruption of cells by treatment with 1 M NaOH and incubation at 80 °C for 5 min.

Quantification of end products. Lactate, acetate and formate were quantified in total NMR sample extracts by ^{1}H-NMR as described elsewhere (Neves *et al.*, 1999). The concentrations of metabolic intermediates and minor end products that remained inside the cells (PEP and 3-PGA) were determined by ^{13}C-NMR. The concentration of labelled lactate determined by ^{1}H-NMR was used as a standard to calculate the concentration of the other metabolites in the sample. Carbon balances from glucose were always above 95 % after taking into account both extracellular and intracellular metabolites.

Quantification of intracellular metabolites in living cells by ^{13}C-NMR. Due to the fast pulsing conditions used for acquiring *in vivo* ^{13}C-spectra, there is no direct correlation between concentrations and peak intensities. The correction factors that allowed the conversion of peak areas of glucose 6-phosphate (G6P), FBP, 3-PGA, NAD^{+} and NADH into intracellular concentrations were determined as described previously (Neves *et al.*, 1999, 2002a). The quantitative kinetic data for intracellular metabolites were calculated from their peak areas by applying the correction factors and comparing with the intensity of the lactate resonance in the last spectrum of the sequence. Intracellular metabolite concentrations were calculated using a value of 2·9 ml (mg protein)$^{-1}$ for the intracellular volume (Poolman *et al.*, 1987).

NMR spectroscopy. All NMR experiments with living cells were run at 30 °C with a quadruple-nucleus probe head on a Bruker DRX500 spectrometer as described before (Neves *et al.*, 1999, 2002b). Although individual experiments are illustrated in each figure, each type of *in vivo* NMR experiment was repeated at least twice and the results were highly reproducible. The values reported are means of two or three experiments and the accuracy varied from 2 % (for end products) to 15 % (for intracellular metabolites with concentrations below 5 mM). The concentration limit for detection of intracellular metabolites in living cells was around 0·6 mM in the experiments where the pools of nicotinamide nucleotides were monitored (2·2 min acquisition time for each spectrum), and 3–4 mM in the remaining experiments (30 s acquisition).

Quantification of the pools of phosphorylated metabolites in growing cells and measurements of PK activity by ^{31}P-NMR were performed as described previously (Ramos *et al.*, 2001). Carbon and phosphorus chemical shifts were referenced to the resonances of external methanol or 85 % H_3PO_4 designated at 49·3 p.p.m. and 0·0 p.p.m., respectively.

Preparation of crude cell extracts for determination of enzyme activities. Cells were cultivated in defined medium as described above until mid-exponential growth phase, harvested, washed and suspended in 50 mM MOPS buffer pH 6·5. Cell extracts were prepared by mechanical disruption using a French press (three passages at 120 MPa), debris was removed by centrifugation (30 000 ***g***, 20 min, 4 °C), and the extracts were used immediately for determination of enzyme activity. The protein concentration in crude cell extracts was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Purification of PK. PK was purified to electrophoretic homogeneity from approximately 65 g (wet wt) of cells of the NZ9000(pNZ*pyk*) strain essentially as described by Crow & Pritchard (1976).

Enzyme assays. PK was routinely assayed in cell extracts by the spectrophotometric method described by Crow & Pritchard (1976). LDH, NADH oxidase (Garrigues *et al.*, 1997) and PFK (Fordyce *et al.*, 1982) were measured as described before.

The effect of activators and inhibitors of PK was determined by ^{31}P-NMR spectroscopy using the purified enzyme. The 3 ml reaction mixtures contained 100 mM Tris/HCl buffer pH 7·5, 5 mM $MgCl_2$, 10 mM KCl, 5 mM FBP, 2 mM ADP and 50 μg pure enzyme. The reactions were started by addition of 2 mM PEP and the time-course for its consumption was monitored; the effect of P_i, FBP, NADH, NAD^{+}, 3-PGA and ATP on the activity of PK was studied by adding these compounds to the reaction mixtures. The reactions were stopped before PEP exhaustion by incubation at 80 °C for 5 min and the rate of PEP consumption was calculated by comparison of the area of the PEP resonance to that of a known amount of methylphosphonate added as an internal standard. One unit of enzyme activity was defined as the amount of enzyme catalysing the conversion of 1 μmol PEP min^{-1} under the experimental conditions used.

All the determinations were made at least in triplicate in two extracts obtained from independent cultures.

RESULTS

Overexpression of the lactococcal PK gene (*pyk*)

The *pyk* gene of *L. lactis* MG1363 was cloned under the control of a nisin-inducible promoter in plasmid pNZ*pyk*, and overexpressed in *L. lactis* NZ9000 by addition of nisin during growth (see details in Methods). In chemically defined medium, a 15-fold higher level of active PK [27·6 units (mg protein)$^{-1}$] was obtained in strain NZ9000(pNZ*pyk*) after induction with nisin (1 ng ml^{-1}), whereas the activity found in NZ9000 harbouring the vector pNZ8020 was similar to that detected in the model organism MG1363 [1·8 units (mg protein)$^{-1}$].

Pools of glycolytic intermediates monitored by *in vivo* NMR

Strains NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) were grown in defined medium containing 1 ng nisin ml^{-1}, harvested in the mid-exponential phase, and glucose metabolism was studied *in vivo* by NMR. Lactate production, glucose consumption and glycolytic intermediates during the metabolism of [1-^{13}C]glucose (40 mM) in both strains under an argon atmosphere are shown in Fig. 1(a, b). The conversion of glucose was homofermentative in strains NZ9000(pNZ*pyk*) and NZ9000(pNZ8020), the glucose consumption rates being $0{\cdot}19 \pm 0{\cdot}01$ and $0{\cdot}22 \pm 0{\cdot}02$ μmol min^{-1} (mg protein)$^{-1}$, respectively. The reduction of the glucose consumption rate in NZ9000(pNZ*pyk*) when compared to the reference strain NZ9000(pNZ8020) is noteworthy; a similar trend was observed in a *L. lactis* construct overproducing NADH oxidase under the control of the nisin promoter (Neves *et al.*, 2002a). A striking difference in the profile of depletion of the FBP pool was found. Although the maximal intracellular concentration reached (approx. 45 mM) was similar in both strains examined, the consumption of FBP after glucose exhaustion proceeded at a much higher rate in the PK-overproducing strain. In addition, 3-PGA accumulated to a maximal concentration of 10 mM in NZ9000(pNZ8020), whereas in NZ9000(pNZ*pyk*) this metabolite was always below the *in vivo* detection limit (Fig. 1a, b). It is worth mentioning that 3-PGA was also not visible in the ^{13}C-NMR spectrum of the NMR-sample extract (not shown) obtained as described in Methods, meaning that its intracellular concentration was below 1·8 mM (detection limit). This result was due to the overproduction of PK in NZ9000(pNZ*pyk*) since this strain behaved like NZ9000(pNZ8020) with respect to the pattern of FBP and 3-PGA accumulation when intermediate levels of PK (7 units mg^{-1}) were achieved by lowering the concentration of nisin to 0·25 ng ml^{-1} (data not shown).

ATP and intracellular P_i concentrations as well as the evolution of intracellular pH during the anaerobic metabolism of glucose by NZ9000(pNZ*pyk*) were determined by ^{31}P-NMR under conditions similar to those used for ^{13}C-NMR experiments (Fig. 1c). Upon substrate addition, the concentration of ATP increased from undetectable levels to a maximum of 6 mM. After glucose exhaustion (at $t \approx 15$ min) a sudden rise of intracellular P_i to about 25 mM was followed by a gradual increase in concentration, reaching values in the order of 40 mM, similar to those of cells in the initial stage of the experiment. The intracellular pH rose from 6·2 in starved cells to 7·3 during active glycolysis and decreased slowly after substrate depletion. These results are similar to those obtained for strain MG1363 under equivalent experimental conditions (Neves *et al.*, 2002b).

Fig. 2 shows the data obtained during the metabolism of [6-^{13}C]glucose (40 mM) by both strains under aerobic (oxygen-saturated) atmosphere. The use of glucose labelled on C-6 allowed us to detect and quantify the resonance of G6P, which otherwise was obscured by the intense peaks due to [1-^{13}C]glucose. The rates of glucose consumption by NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) were $0{\cdot}12 \pm 0{\cdot}01$ and $0{\cdot}16 \pm 0{\cdot}01$ μmol min^{-1} (mg protein)$^{-1}$, respectively. Lowered glycolytic fluxes have been consistently observed

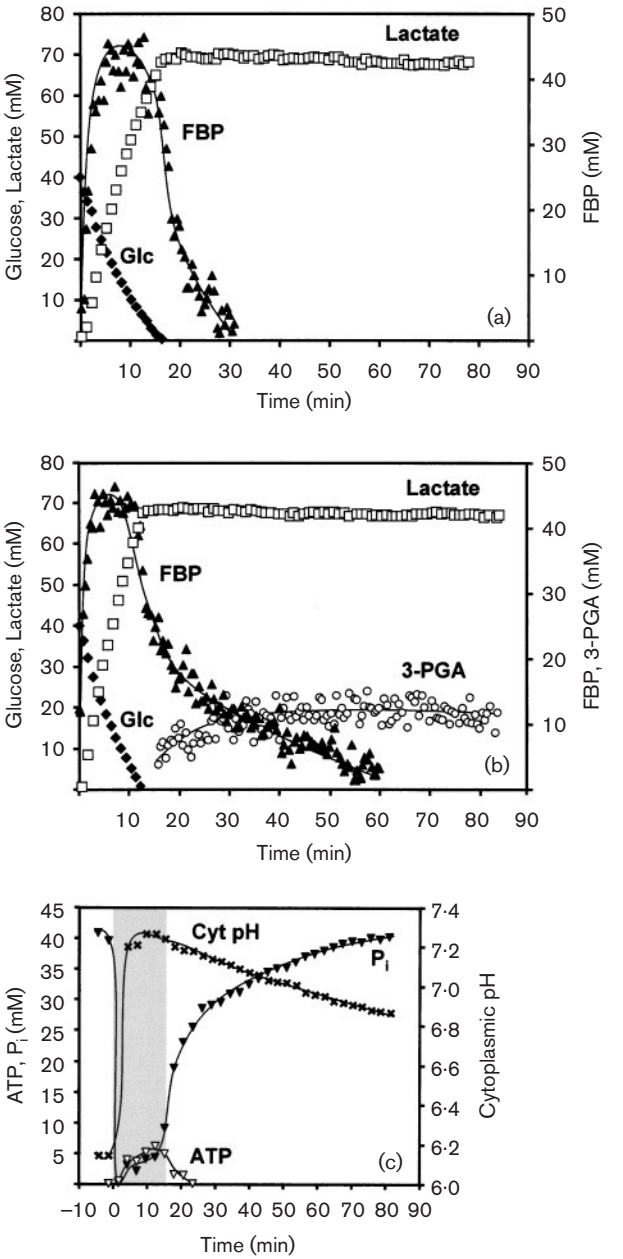

Fig. 1. (a, b) Metabolism of glucose under anaerobic conditions at 30 °C, pH 6·5, assessed by ^{13}C-NMR *in vivo*: time-course for the consumption of [1-^{13}C]glucose (40 mM), lactate formation and evolution of the FBP pool in (a) NZ9000(pNZ*pyk*) and (b) NZ9000(pNZ8020). (c) Intracellular pH, KP_i and ATP concentrations were determined in the PK-overproducer by ^{31}P-NMR after a pulse of glucose using the same experimental conditions. Glucose was added at time 0 and the shaded area indicates glucose availability. ◆, Glucose; □, lactate; ▲, FBP; ○, 3-PGA; ▽, ATP; ▼, P_i; ×, cytoplasmic pH.

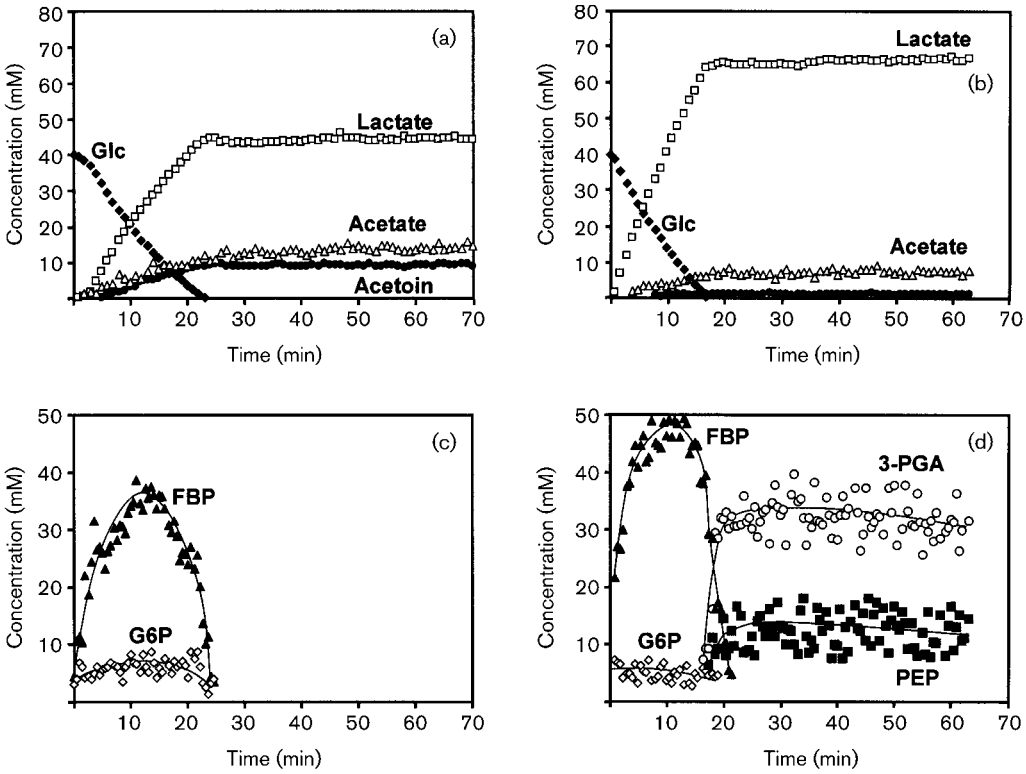

Fig. 2. Effect of oxygen on the metabolism of glucose monitored by *in vivo* ^{13}C-NMR: time-course for the consumption of [6-^{13}C]glucose (40 mM) and end product formation (a, b) and evolution of the intracellular metabolite pools (c, d) in strains NZ9000(pNZ*pyk*) (a, c) and NZ9000(pNZ8020) (b, d). ◆, Glucose; □, lactate; △, acetate; ●, acetoin; ▲, FBP; ◇, G6P; ○, 3-PGA; ■, PEP.

under aerobic conditions in several *L. lactis* strains (Neves *et al.*, 2002a). The behaviour of strain NZ9000(pNZ8020) (Fig. 2b, d) was comparable to that reported for MG1363 under an oxygen atmosphere (Neves *et al.*, 2002b): after the addition of glucose, FBP and G6P rose to approximately 48 and 5 mM, respectively, decreasing to undetectable levels after glucose depletion, whilst 3-PGA and PEP accumulated to concentrations of approximately 32 and 10 mM, respectively, levels that are much higher than those measured under an argon atmosphere. Acetate (7·8 mM) and acetoin (1·1 mM) were produced in addition to lactate (67·2 mM). In the NZ9000(pNZ*pyk*) strain (Fig. 2a, c), the production of acetate and acetoin was, respectively, two- and eightfold higher than in NZ9000(pNZ8020). 3-PGA and PEP were not detected, either *in vivo* or in NMR-sample extracts. The acceleration of the FBP consumption rate occurred in both strains upon switching from anaerobic to aerobic conditions (compare Figs 1 and 2), but the maximal FBP level (approx. 37 mM) in the NZ9000(pNZ*pyk*) strain was clearly lower under an oxygen atmosphere as compared to anaerobic conditions (approx. 45 mM).

Characterization of pyridine nucleotides kinetics during anaerobic glycolysis

Recently, we described the on-line determination of ^{13}C-glycolytic intermediates, NAD$^+$ and NADH in resting cells of strain MG1363 and an LDH-deficient strain (Neves *et al.*, 2002b). A similar approach was followed here to assess the magnitude of the NAD$^+$ and NADH pools in strain NZ9000(pNZ*pyk*) and in strain NZ9000(pNZ8020), harbouring the vector plasmid without the *pyk* insert. The kinetics of consumption of [1-^{13}C]glucose (80 mM), the build-up and depletion of FBP, and the evolution of NAD$^+$ and NADH under an argon atmosphere are shown in Fig. 3. In starved cells of NZ9000(pNZ*pyk*), the concentration of NAD$^+$ was approximately 6 mM. The NAD$^+$ pool decreased steadily during glucose utilization, reaching a minimum at about 3·5 mM. At this stage the pool of NADH was approximately 1·8 mM. Thereafter, NAD$^+$ recovered to its initial concentration while NADH returned to undetectable levels. The time-course for the pyridine nucleotide pools was different in strain NZ9000(pNZ8020). The NAD$^+$ pool was constant while glucose was available but decreased sharply at the onset of glucose depletion, reaching the minimum value of 1·7 mM. Thereafter, NAD$^+$ recovered very slowly. A similar behaviour has been reported for the model strain MG1363 (Neves *et al.*, 2002b).

Quantification of phosphorylated metabolites during growth

Strains NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) were grown under anaerobic conditions at pH 6·5 in defined

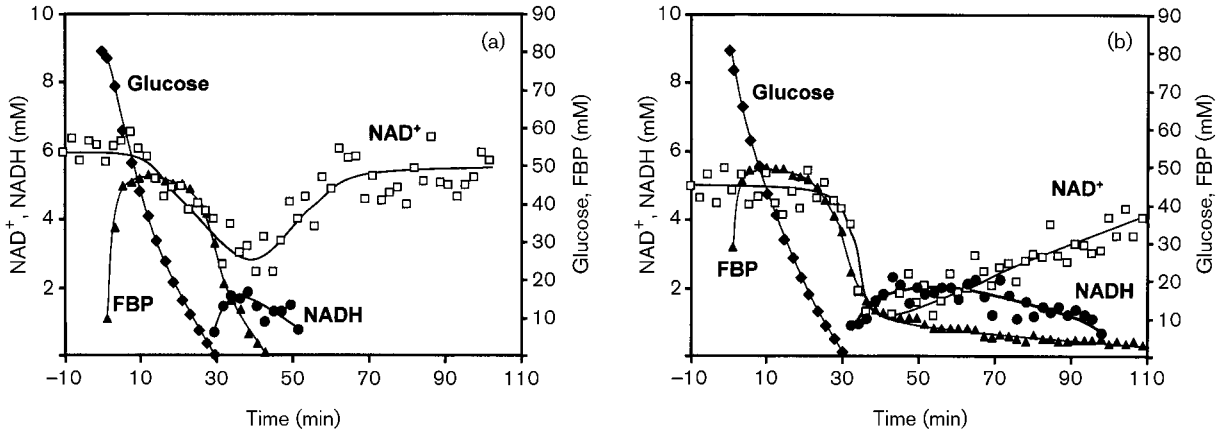

Fig. 3. Kinetics of [1-^{13}C]glucose (80 mM) consumption and build-up/depletion of FBP, NAD^+ and NADH pools, as monitored by *in vivo* ^{13}C-NMR in strains NZ9000(pNZ*pyk*) (a) and NZ9000(pNZ8020) (b) during glycolysis under anaerobic conditions. Glucose was added at time 0. ◆, Glucose; ▲, FBP; □, NAD^+; ●, NADH.

medium containing 1 ng nisin ml^{-1}. The growth rate of NZ9000(pNZ*pyk*) ($0{\cdot}42 \pm 0{\cdot}03\ h^{-1}$) was consistently lower than that of NZ9000(pNZ8020) ($0{\cdot}51 \pm 0{\cdot}02\ h^{-1}$). Ethanol extracts were prepared from culture samples in the mid-exponential (OD_{600} 2·2) and stationary (OD_{600} 4·5) growth phases. Fig. 4 shows the pools of phosphorylated glycolytic metabolites determined in cell extracts by ^{31}P-NMR. The profile of glycolytic intermediates in the mid-exponential phase was similar in both strains examined, the major metabolites being G6P and FBP. Extracts obtained from NZ9000(pNZ8020) in the stationary growth phase contained 3-PGA (23 mM), 2-PGA (2·6 mM) and PEP (7·1 mM), the metabolites typical of starvation in *L. lactis*, whereas in NZ9000(pNZ*pyk*) none of these metabolites accumulated above the detection limit.

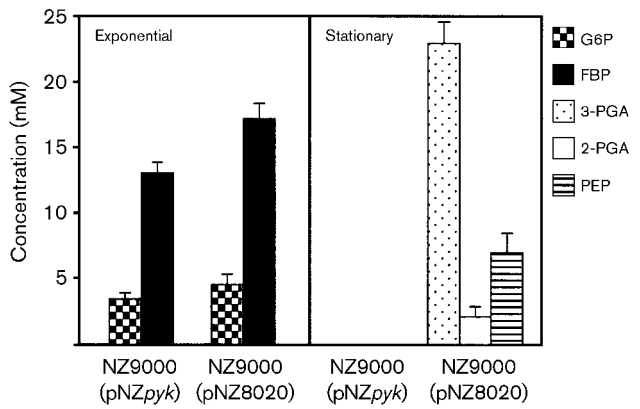

Fig. 4. Concentrations of phosphorylated metabolites during growth. Strains NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) were grown in defined medium containing 1 % (w/v) glucose, 1 ng nisin ml^{-1} and 5 μg chloramphenicol ml^{-1}, at pH 6·5 and 30 °C. Ethanol extracts were obtained in the mid-exponential or stationary growth phases as described in Methods. The data shown are means of three independent determinations.

Determination of enzyme activities

The activity of LDH, PK, PFK and NADH oxidase was assessed in extracts obtained from cultures of NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) growing under either anaerobic or aerobic atmosphere. The genes *pfk*, *pyk* and *ldh* encoding PFK, PK and LDH constitute the *las* operon (Llanos *et al.*, 1993). Therefore, to assess whether the utilization of the nisin transcription induction system affects indirectly expression of the *las* operon, LDH, PK and PFK activities were also measured in extracts of the parental strain NZ9000 (Table 1). The activity of NADH oxidase was similar in NZ9000(pNZ*pyk*) and NZ9000(pNZ8020) and, as expected, it was induced by growth in the presence of oxygen. However, the activity of LDH was significantly lower in the PK-overproducing strain. The LDH specific activity decreased by about 50 % in NZ9000(pNZ*pyk*) grown under anaerobic conditions when compared to NZ9000(pNZ8020) or NZ9000, which exhibited similar levels of LDH. This effect was even more pronounced in extracts of the overproducer strain grown under an oxygen atmosphere. The activity of PFK was reduced by approximately 50 % in extracts of the two strains carrying plasmids [NZ9000(pNZ*pyk*) and NZ9000(pNZ8020)] when compared to NZ9000.

The effect of several effectors, such as P_i, NAD^+, NADH, 3-PGA and ATP, on the activity of PK (pure enzyme) was assessed by ^{31}P-NMR. The activity was not affected by NADH or NAD^+, added at concentrations of 2 or 5 mM, respectively; however, ATP and 3-PGA (added at a concentration of 10 mM) caused a reduction in PK activity to 30 and 60 %, respectively (data not shown). Under the experimental conditions used here, the concentration of P_i required to give 50 % inhibition was 1·5 mM, a value comparable to that determined for the lactococcal enzyme

Table 1. Enzyme activities in extracts of *L. lactis* NZ9000 and derivative strains grown under anaerobic or aerobic conditions

Assays were performed under non-controlled atmosphere. Enzyme activities are reported as units (mg protein)$^{-1}$ ± standard deviation ($n \geqslant 4$). ND, Not determined.

Enzyme	NZ9000(pNZ*pyk*)		NZ9000(pNZ8020)		NZ9000
	Argon	Oxygen	Argon	Oxygen	Argon
PFK	0·87 ± 0·11	ND	0·85 ± 0·10	ND	1·5 ± 0·2
PK	27·6 ± 0·8	25·2 ± 0·7	1·8 ± 0·1	1·5 ± 0·1	1·5 ± 0·2
LDH	15·7 ± 0·4	3·8 ± 0·2	28·0 ± 0·6	11·6 ± 1·0	31·9 ± 1·3
NADH oxidase	0·07 ± 0·0	0·14 ± 0·01	0·05 ± 0·0	0·15 ± 0·02	ND

with the conventional NADH-coupled assay (Crow & Pritchard, 1976).

DISCUSSION

The complexity of metabolic and regulatory networks is a great challenge to an integrated view of how individual components contribute to the overall cell function even in an organism with the apparent metabolic simplicity of *L. lactis*. NMR spectroscopy is a useful methodology since metabolic changes can be monitored in real time under physiological conditions. Moreover, as *in vivo* NMR experiments are usually performed with resting cells, anabolism is highly depressed, and the metabolic network simplified to a great extent. Recently, ^{13}C labelling coupled to NMR detection provided a description of the dynamic behaviour of glucose, end products, pools of glycolytic intermediates, NAD^+ and NADH, and showed that the pool of reduced pyridine nucleotides is not a primary factor in the regulation of the glycolytic flux in resting cells of *L. lactis* (Neves *et al.*, 2002b).

The present work takes advantage of a similar experimental approach to provide a detailed picture of glycolysis in a strain overproducing PK, a key glycolytic enzyme assumed to play a major role in the regulation of glycolysis. The most notable consequences of the overproduction of PK were the acceleration of the rates of FBP depletion and NAD^+ recovery once glucose was exhausted, and the lack of accumulation of 3-PGA and PEP, the two metabolites associated with cell starvation. In strain NZ9000(pNZ8020), the level of FBP decreased very slowly after reaching the intracellular level of about 20 mM, a profile identical to that of strain MG1363, the parent strain of NZ9000 (Neves *et al.*, 2002b). This observation denotes a constriction at the level of PK, further evidenced by the accumulation of metabolites immediately upstream of pyruvate, namely 3-PGA and PEP (Neves *et al.*, 2002a). The higher rates of FBP depletion and of NAD^+ recovery, and the absence of detectable pools of 3-PGA or PEP in the PK-overproducing strain, suggest the occurrence of a metabolic bottleneck at the level of PK in NZ9000(pNZ8020) when glucose became limiting; in the PK overproducer this constriction was no longer present, allowing, in addition to a rapid FBP consumption, a complete carbon flow to lactate, thus preventing accumulation of 3-PGA and PEP. The obstruction at the level of PK is primarily ascribed to accumulation of P_i, a well-known inhibitor of the enzyme, counteracting the activating properties of FBP (Crow & Pritchard, 1976, 1982). In fact, when glucose transport stopped, the concentration of P_i rose abruptly due to the release of phosphate engaged mainly in the large FBP pool and other phosphorylated metabolites (Fig. 1). The data provide clear evidence for the role of P_i as an important regulator of the flux through PK *in vivo*.

The absence of 3-PGA and PEP (below the detection limit of the technique) is even more striking when aerobic conditions are considered (Fig. 2c) since this and earlier studies have shown that the levels of these metabolites are greatly increased in the presence of oxygen (Fig. 1b versus Fig. 2d and Neves *et al.*, 2002a). In strain NZ9000(pNZ8020) approximately 50 % of the FBP pool was converted to 3-PGA and PEP (Fig. 2d), meaning that the amount of carbon processed via PK, once glucose was depleted, was low. However, this bottleneck at the level of PK was completely overcome by 15-fold overproduction of PK (Fig. 2c).

It has been shown that the NAD^+ pool is maintained at the maximum level in MG1363 when glucose is actively metabolized (Neves *et al.*, 2002b). Surprisingly, the level of NAD^+ decreased gradually during glucose consumption in the PK overproducer, indicating that NADH reoxidation was less efficient in this strain. We think that this is caused by the depression of the LDH activity in NZ9000(pNZ*pyk*), an unexpected lateral effect of the genetic manipulation strategy pursued here. One could speculate that the nisin induction system exerts a deleterious effect on the general transcription machinery and indirectly affects expression of the *pfk–pyk–ldh* (*las*) operon. However, this explanation is not satisfactory since, at least under anaerobic conditions, the PK and LDH activities of NZ9000 were not appreciably affected by the presence of the vector plasmid. In addition, the low levels of LDH in strain NZ9000(pNZ*pyk*) could not be due to downregulation of transcription of the *las* operon mediated by PK overexpression, since the levels of PFK were similar in both engineered strains used in the present work (Table 1). Surprisingly, Andersen *et al.* (2001a) observed

that alterations of the *las* promoter affect differentially the expression of the three genes of the operon. One explanation could be that the *las* operon is subjected to posttranscriptional regulation, which results in alterations of its mRNA fate and consequently modulates the translation efficiency of its genes. This view is supported by the observation of processed species of the *las* mRNA (Luesink *et al.*, 1998). If this is the case, the presence of chloramphenicol (a translation inhibitor) during growth of cultures carrying plasmids could differentially affect expression of *pfk* and *ldh*. Moreover, the pronounced inhibitory effect in the synthesis of LDH could explain the lower growth rate of NZ9000(pNZ*pyk*) than that of NZ9000(pNZ8020).

The level of LDH in NZ9000(pNZ*pyk*) was reduced to approximately 50 % of that of NZ9000, and this may be sufficient to create a bottleneck at the level of LDH and hinder the regeneration of NAD^+. In fact, according to Andersen *et al.* (2001b), *L. lactis* has limited excess capacity of LDH, only 70 % more than needed to sustain the lactate flux in the wild-type cells. Interestingly, despite the lower NAD^+/NADH ratio observed in the PK-overproducing strain, glucose was metabolized largely to lactate as in the wild-type strain. Therefore, it seems that the redistribution of carbon flux in the pyruvate node does not depend critically on the level of NAD^+, and the NAD^+/NADH ratio can vary to some extent without causing noticeable changes in the composition of end products.

The decreased LDH activity was probably also the main reason for the shift towards acetate and acetoin production, under aerobic conditions. The production of acetate and acetoin was considerably higher in the PK-overproducer than in NZ9000(pNZ8020), since the activity of LDH was approximately threefold lower in the former strain while NADH oxidase activity was similar in both strains.

Overexpression of the gene encoding PK, a postulated regulatory enzyme, did not lead to an increased glycolytic flux in non-growing cells, instead a slight decrease was observed; also, the growth rate of the PK-overproducer was reduced (17 %). It is worth mentioning that the patterns of glycolytic intermediates in growing cells (Fig. 4) were similar to those measured by *in vivo* NMR in non-growing cell suspensions, namely the absence of 3-PGA in the PK-overproducer after glucose depletion. Lower growth and glucose consumption rates in this mutant may be a consequence of (i) diminished level of LDH, and/or (ii) protein burden, as observed in *Zymomonas mobilis* (Snoep *et al.*, 1995) and *E. coli*, where overproduction of PK or PFK led to reduced glucose bioconversion rates relative to the control (Emmerling *et al.*, 1999).

A recent study using metabolic control analysis excluded a role in the control of flux for the enzymes encoded by the *las* operon in growing cells of *L. lactis* MG1363 (Koebmann *et al.*, 2002b). The same laboratory has reported that the demand for ATP exerts a strong control on the glycolytic flux of resting cells (Koebmann *et al.*, 2002a). We found that the glycolytic flux in resting cells of *L. lactis* did not increase in response to a 15-fold increase in the level of PK, but when glucose became limiting, PK overproduction allowed a greater flux through PK and led to complete channelling of carbon to end products without accumulation of the glycolytic intermediates typical of starvation.

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