Transcriptional, translational and metabolic regulation of glycolysis in *Lactococcus lactis* subsp. *cremoris* MG 1363 grown in continuous acidic cultures

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The physiological behaviour of *Lactococcus lactis* subsp. *cremoris* MG 1363 was characterized in continuous culture under various acidic conditions (pH 4.7–6.6). Biomass yield was diminished in cultures with low pH and the energy dedicated to maintenance increased due to organic acid inhibition and cytoplasmic acidification. Under such acidic conditions, the specific rate of glucose consumption by the bacterium increased, thereby enhancing energy supply. This acceleration of glycolysis was regulated by both an increase in the concentrations of glycolytic enzymes (hierarchical regulation) and the specific modulation of enzyme activities (metabolic regulation). However, when the inhibitory effect of intracellular pH on enzyme activity was taken into account in the model of regulation, metabolite regulation was shown to be the dominant factor controlling pathway flux. The changes in glycolytic enzyme concentrations were not correlated directly to modifications in transcript concentrations. Analyses of the relative contribution of the phenomena controlling enzyme synthesis indicated that translational regulation had a major influence compared to transcriptional regulation. An increase in the translation efficiency was accompanied by an important decrease of total cellular RNA concentrations, confirming that the translation apparatus of *L. lactis* was optimized under acid stress conditions.

INTRODUCTION

*Lactococcus lactis* is a neutrophilic bacterium with an optimum pH for growth of between 6·3 and 6·9 (Hutkins & Nannen, 1993). This micro-organism converts sugars into lactic acid, thus provoking medium acidification, a characteristic of economic importance within the dairy industries. The behaviour of *L. lactis* in acidic conditions can considerably modulate the characteristics of fermented foods. Indeed, during cheese manufacture, the capacity of *L. lactis* to grow, retain active catabolism and survive at low pH directly influences the final pH of the product and flavour production.

Some data concerning the cellular response of *L. lactis* to acidification and acid stress are now available, but the picture is still incomplete. *L. lactis* develops a complex adaptive response to acid stress, which is dependent on the synthesis of proteins such as heat-shock proteins and proteinases (Rallu et al., 1996; Hartke et al., 1996; Kim et al., 1999; O’Sullivan & Condon, 1997, 1999). Although the synthesis of proteins by *L. lactis* is often studied, metabolic aspects of the *L. lactis* stress response are generally not investigated under acidic conditions. However, it is known that the drop in pH related to lactic acid production leads to a progressive inhibition of growth and ultimately to growth arrest at approximately pH 4, corresponding to an intracellular pH (pHin) of 5·4–5·9 (Hutkins & Nannen, 1993; O’Sullivan & Condon, 1997; Mercade et al., 2000). Recently, the dynamic response of catabolic pathways of *L. lactis* was studied during auto-acidification (Even et al., 2002). However, in this work, the rapid acidification of the culture medium led to the onset of stationary phase and only the post-acidification phase was characterized completely. It remains to be determined how growth of *L. lactis* can continue under unfavourable pH conditions and the extent to which the various regulations are operative in such acidic environments.

To examine the effect of acidic conditions on the growth of *L. lactis*, the behaviour of growing cells of *L. lactis* subsp. *cremoris* MG 1363 was characterized in continuous cultures under various controlled-pH steady-states. This approach is particularly adapted to the study of acid stress since a constant growth rate can be imposed, thus allowing the cellular response to pH to be studied independently of the
changes to the growth rate that often accompany medium acidification in batch cultures. The metabolic pathways responsible for lactic acid production, which causes the acid stress, have been examined by integrating metabolic, energetic and transcriptional profiles. These pathways are essential to *L. lactis* as they provide the energy necessary for growth and for energy-dependent mechanisms involved in the acid stress response, such as ATPase. This work contributes to the overall understanding of the metabolic modifications induced at low pH in *L. lactis*, whose essential role in the food industry is directly related to the acidification phenomenon.

### METHODS

**Organism and growth conditions.** *L. lactis* subsp. cremoris MG 1363 was used throughout this work; this bacterium lacks the lactose plasmid (Gasson, 1983). The strain was grown on simplified synthetic medium MS10 (Cocaign-Bousquet et al., 1995) supplemented with glucose (10 g l\(^{-1}\)) as carbon source.

Continuous cultures were performed under anaerobic conditions, in a nitrogen atmosphere, in a 400 ml fermenter (Midiverre Laboratoire) at 30 °C. Regulation of pH was achieved by automatic addition of KOH (10 M). For pH ≤6-6, the proportions of KH\(_2\)PO\(_4\) and K\(_2\)HPO\(_4\) were modified in the feed medium so as to use medium flowing into the reactor at the desired pH. Each steady-state culture was obtained from an independent pre-culture to avoid progressive adaptation of the strain. Continuous cultures were maintained until constant biomass and fermentation profiles were observed, and steady-state sampling was performed after 4 days (10 dilution times).

For the growth-inhibition profile induced by sodium lactate, cells were grown in butyl-rubber-stoppered tubes on MS10 medium (pH 6-6 or 4-7) supplemented with glucose (10 g l\(^{-1}\)) and DTT (1 mM) and different amounts of sodium lactate. The initial pH was adjusted to 6-6 or 4-7 with KOH (10 M) after the addition of lactate.

**Fermentation analysis.** Bacterial growth was monitored spectrophotometrically at 580 nm and calibrated against cell-dry-weight measurements. A change of 1 U was shown to be equivalent to 0.28 g dry matter l\(^{-1}\). Glucose and fermentation products (lactate, formate, acetate and ethanol) and amino acids were determined by HPLC as described previously (Novak et al., 1993). A mixture that contained 200 μl triethanolamine buffer (500 mM, pH 7-6, containing 15 mM MgSO\(_4\) and 4 mM EDTA), 20 μl of 3-phosphoglycerate (250 mM), 25 μl NADH (0-5 mM), 20 μl glyceraldehyde-3-phosphate dehydrogenase (500 U ml\(^{-1}\)), 400 μl extract and 335 μl H\(_2\)O was prepared; 20 μl of 3-phosphoglycerate kinase (1000 U ml\(^{-1}\)) were added to the mixture to initiate ATP consumption. All measurements were obtained relative to cell dry weight but were expressed as aqueous molar values, using the mean intracellular volume of 3 ml g\(^{-1}\) determined by the method of Uribelarra et al. (1985).

**ATP measurement.** Intracellular ATP was extracted as described previously (Garrigue et al., 1997) and measured by the method developed by Le Bloas et al. (1993). A mixture that contained 200 μl triethanolamine buffer (500 mM, pH 7-6, containing 15 mM MgSO\(_4\) and 4 mM EDTA), 20 μl of 3-phosphoglycerate (250 mM), 25 μl NADH (0-5 mM), 20 μl glyceraldehyde-3-phosphate dehydrogenase (500 U ml\(^{-1}\)), 400 μl extract and 335 μl H\(_2\)O was prepared; 20 μl of 3-phosphoglycerate kinase (1000 U ml\(^{-1}\)) were added to the mixture to initiate ATP consumption. All measurements were obtained relative to cell dry weight but were expressed as aqueous molar values, using the mean intracellular volume of 3 ml g\(^{-1}\) determined by the method of Uribelarra et al. (1985).

**Preparation of crude extract and enzyme assays.** A volume of culture corresponding to 58 mg (dry weight) of cells was centrifuged (4 °C, 10 min at 6000 g) and washed twice with 0-2% (v/v) KCl. Cells were resuspended in Tris (45 mM)/tricarballylate (15 mM) buffer (pH 7-2) containing glycerol (20%, v/v), MgCl\(_2\) (4-5 mM) and DTT (1 mM). Cell disruption by sonication (5 cycles of 30 s interspersed with 1 min cooling periods) was followed by the removal of cell debris by centrifugation (4 °C, 10 min at 6000 g).

The supernatant was used for all enzyme assays. The protein concentration of enzymic extracts was determined by the Lowry method, with BSA as the standard. Specific enzyme activities [nmol (mg protein\(^{-1}\)] were converted to whole-cell activities [nmol (g dried cells\(^{-1}\) h\(^{-1}\)] using the measured protein content of *L. lactis*, which was found to be constant at 42% of dry weight during each steady-state. All enzymes were assayed immediately after cell disruption at 30 °C and pH 7-2 using previously described assay procedures (Even et al., 2001).

**Determination of pH-dependent enzyme activity profiles.** The pH activity profiles were determined in Tris (50 mM)/acetate (12-5 mM) buffer. Activities were assayed in substrate and co-substrate saturating concentrations and with excess amounts of coupling enzymes. Controls were carried out to ensure that the coupling enzymes were never rate-limiting.

**Proton motive force.** Proton gradient (∆pH) and electric gradient (∆Ψ) values were measured by determining the internal-to-external gradient of \([\text{14C}]\)benzoic acid and \([\text{3H}]/\text{tetraphenylphosphonium bro- mide, respectively, after centrifugation of the cells through silicon oil as described previously (Loubiere et al., 1992).**

**Handling of RNA and transcript labelling.** A volume of culture (corresponding to 6 mg dry weight cells) was harvested, centrifuged (4 °C, 5 min at 8000 g), washed with 1 ml TE buffer [Tris/HCl (10 mM, pH 8), EDTA (1 mM)], resuspended in 500 μl TE buffer and frozen immediately in liquid nitrogen. Cells were stored at −80 °C until extraction.

As described previously (Fontaine et al., 2001; Even et al., 2001, 2002), total RNA was extracted using a variant of the acid/phenol method and quantified spectrophotometrically; 2 μg of this RNA was then chemically labelled with digoxigenin and stored at −20 °C until hybridization.

**Preparation of macro-arrays and hybridization.** PCR-based macro-arrays that included all the glycolytic genes were made on nylon membranes as described previously (Fontaine et al., 2001; Even et al., 2001). Pre-hybridization, hybridization and washing steps were performed as described by Fontaine et al. (2001).

**Array detection and analysis.** Membranes were incubated in a solution containing anti-digoxigenin conjugate coupled to alkaline phosphatase (75 μU ml\(^{-1}\)) (Roche). The alkaline phosphatase substrate AttoPhos (Amersham Pharmacia Biotech) was then added and the membranes were scanned with a phosphofluoroimager (STORM 860, Molecular Dynamics) as described previously (Fontaine et al., 2001; Even et al., 2001). Quantification was performed at different times after the addition of AttoPhos, leading to the calculation of the alkaline phosphatase activity for each slot. Alkaline phosphatase activity correctly calculated in its linearity domain is directly correlated to the mRNA hybridized on the slot probe. Since the total RNA concentration was normalized in the assay (2 μg), the alkaline phosphatase activity revealed the abundance of a specific messenger in the total RNA population. This value was corrected using the cellular RNA concentration, which is known to vary with the environmental conditions, in order to obtain the cellular concentration of a specific messenger as described previously (Fontaine et al., 2001; Even et al., 2001, 2002). Results were standardized using the values obtained from the steady-state culture at pH 6-6 and expressed as expression ratios.

To evaluate the reproducibility of this method, four RNA samples taken from the exponential phase of a batch culture — i.e. the cells were in the same physiological state — were analysed in parallel. The standard deviation value was found to be approximately constant for the various genes of glycolysis and the mean standard deviation value for the mRNA cellular concentrations was found to be ±36%
(Fontaine et al., 2001; Even et al., 2001). As done previously (Even et al., 2001, 2002), expression ratios were considered to be significantly different when differing by at least two standard deviations (i.e. \( \leq 0.6 \) or \( \geq 1.7 \)). In these conditions, the probability that the observed changes for a transcript are real and not due to experimental error is 95%.

**RESULTS**

**Kinetic parameters**

*L. lactis* subsp. *cremoris* MG 1363 was grown in steady-state chemostat cultures on MS10 medium containing 55 mM glucose at four different pH values (6·6, 5·5, 5·2 and 4·7). A dilution rate of 0·1 h\(^{-1}\) was used, enabling the bacteria to sustain growth despite pH inhibition. Biomass, glucose and product concentrations were measured in the four steady-states (Fig. 1). Biomass concentration was drastically affected in acidic conditions and diminished linearly with the imposed pH. At pH values greater than 4·7, cell growth was strictly carbon-limited with no detectable residual glucose, but at pH 4·7 a significant amount of glucose accumulated in the medium (30 mM). A progressive shift towards homolactic metabolism was seen as the steady-state pH was diminished, along with a decrease in the amount of mixed-acid metabolites (formate, acetate and ethanol) that accumulated in the medium and an increase in the proportion of pyruvate converted to lactate (18% at pH 6·6 to 96% at pH 4·7).

Specific rates of glucose consumption and product formation were calculated from the steady-state concentrations (Table 1). The specific rate of glucose consumption \((q_g)\) increased when the pH decreased from 6·6 to 5·2 and although the \(q_g\) fell slightly at pH 4·7 it remained significantly higher than at pH 6·6. Changes in the specific rate of lactate formation were even more pronounced due to the metabolic shift towards homolactic fermentation as the pH was decreased. In accordance with this metabolic shift, specific rates of formate, acetate and ethanol production were diminished in acidic conditions.

**Amino acid consumption**

Significant residual amounts of the various amino acids were detected in the four steady-states (data not shown), except for arginine, which was exhausted at pH 6·6. Specific overall rates of amino acid consumption were calculated and the sums of them (expressed as carbon equivalents) are presented in Table 1. Amino acid consumption remained constant at all steady-states except for that at pH 4·7, for

![Fig. 1. Concentrations of biomass (●), glucose (○), lactate (■), formate (△), acetate (▲) and ethanol (▼) during steady-state continuous culture of *L. lactis* subsp. *cremoris* MG 1363 on MS10/glucose medium at a growth rate of 0·1 h\(^{-1}\) and different pH values.](image)

### Table 1. Specific rates of glucose \((q_g)\) and amino acid \((\Sigma_{A_{AA}})\) consumption, lactate \((v_L)\), formate \((v_F)\), acetate \((v_A)\) and ethanol \((v_E)\) formation, and carbon recovery during continuous culture of *L. lactis* subsp. *cremoris* MG 1363 on MS10/glucose medium at a growth rate of 0·1 h\(^{-1}\) and different pH values

<table>
<thead>
<tr>
<th>Parameter [unit(s)]</th>
<th>Specific rate at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4·7</td>
</tr>
<tr>
<td>(q_g) [mmol (g cells(^{-1}) h(^{-1})]</td>
<td>8·9 ± 0·6</td>
</tr>
<tr>
<td>(v_L) [mmol (g cells(^{-1}) h(^{-1})]</td>
<td>17·7 ± 0·8</td>
</tr>
<tr>
<td>(v_F) [mmol (g cells(^{-1}) h(^{-1})]</td>
<td>0·7 ± 0·1</td>
</tr>
<tr>
<td>(v_A) [mmol (g cells(^{-1}) h(^{-1})]</td>
<td>0·2 ± 0·2</td>
</tr>
<tr>
<td>(v_E) [mmol (g cells(^{-1}) h(^{-1})]</td>
<td>&lt;0·05</td>
</tr>
<tr>
<td>(\Sigma_{A_{AA}}) [mmol C (g cells(^{-1}) h(^{-1})]*</td>
<td>33·9 ± 6</td>
</tr>
<tr>
<td>Carbon recovery (%)†</td>
<td>66 ± 9</td>
</tr>
</tbody>
</table>

*Sums of the specific rates of consumption of each amino acid are expressed as carbon equivalents.
†Calculated by integrating glucose and amino acid consumption and biomass and identified product formation with a biomass molecular mass of 129 g mol\(^{-1}\) (C\(_{4·63}\)H\(_{7·89}\)O\(_{2·53}\)N). Carbon recovery = \(\frac{3\times (v_L + v_F + v_A + v_E)}{4630/129 + \sum v_{AA}}\) × 100 with \(D\), dilution rate (h\(^{-1}\)).

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which a fivefold increase was observed. Indeed, at pH 4–7, amino acid consumption represented 39% of the total carbon consumed, as compared to 11–15% for higher pH values. Carbon recovery, calculated by integrating glucose and amino acid consumption and biomass and product formation, was close to 100% for the steady-states at pH 5–2 and higher, but diminished to only 66% at pH 4–7. This carbon imbalance at pH 4–7 indicates the accumulation of unidentified products and, since at this pH the consumption of amino acids was significantly increased, it is postulated that these unidentified products are derived from amino acid degradation. Indeed, in acidic conditions, amino acid catabolism (deamination, decarboxylation) is known to occur (Christensen et al., 1999; Sanders et al., 1999). To confirm the presence of amino acid catabolism at pH 4–7, the concentration of NH₃ was measured in the different steady-states; a sixfold increase in the specific rate of NH₃ production was found at pH 4–7 compared to pH 6–6 [i.e. 1·2 mmol (g cells)−¹ h−¹ at pH 4–7 compared to 0·2 mmol (g cells)−¹ h−¹ at pH 6·6].

Identification of the growth-limiting factor at pH 4–7

While cells of *L. lactis* subsp. *cremoris* MG 1363 were clearly carbon-limited at pH values of 5·2 and higher (no residual glucose detected), less than half the supplied glucose was consumed at pH 4·7. Supplementing the inflowing medium with the various components of the medium (glucose, amino acids, vitamins and salts) did not modify the steady-state parameters at pH 4–7, indicating that the appearance of residual glucose could not be attributed to a change in the growth-limiting nutrient (data not shown). On the contrary, addition of lactate (25 mM) directly into the fermenter resulted in cell washout until the lactate concentration was re-established at the initial concentration. The steady-state was therefore dependent on the lactic acid inhibition phenomenon at pH 4–7. To characterize this inhibition better, the maximum growth rate of the strain was investigated in batch cultures in the presence of various amounts of sodium lactate and at pH 4–7 and 6·6 (Fig. 2). Inhibition by lactate was observed to be considerably more intense under acidic conditions, as illustrated by the decrease of the inhibition constant from 470 mM at pH 6·6 to 13 mM at pH 4–7. Furthermore, at pH 6·6, the critical concentration at which growth no longer possible was 710 mM lactate compared to 87 mM at pH 4·7.

Energetic parameters

Steady-state growth at low pH led to an increase in the specific rate of ATP formation, in proportion to the specific rate of glucose consumption (Table 2). ATP formation was also associated with arginine catabolism through the arginine deiminase pathway, which was quantified here by ornithine production. However, this ATP production was always negligible compared to the ATP generated from glucose catabolism. The biomass yield relative to ATP production (*Y*<sub>ATP</sub>) decreased as the pH was diminished from 6·6 to 5·2. For the steady-state at pH 4·7, a slight increase in ATP production was observed, although this was probably due to additional ATP-generating reactions associated with amino acid catabolism not included in the *Y*<sub>ATP</sub> estimation. The pH<sub>in</sub> decreased as a function of the extracellular pH but this decrease in internal pH was less important than that of the extracellular pH due to an increase of the ΔpH at pH values other than 6·6 (Table 2). The ΔΨ did not show any appreciable variations in cells from the steady-states at pH 6·6 to 5·2, but was significantly higher at pH 4·7. As a consequence, the proton motive force increased markedly at pH 4·7. The ATP pool was also quantified and shown to increase progressively as the steady-state pH was diminished.

Enzymic activities

Specific activities of all enzymes of glycolysis were assayed in steady-state cells (Table 3). Most enzymic activities increased in acidic conditions compared to pH 6·6, even if at pH 4·7 a slight decrease in activity was often observed. Only glyceraldehyde-3-phosphate dehydrogenase activity was seen to diminish as a function of the pH.

Since enzyme activities were measured *in vitro* at pH 7·2, the values are indicative of intracellular enzyme concentrations but do not take into account the activity that might be functional within the cell at the different pH<sub>in</sub> values observed. Therefore, the effect of pH on the various activities was quantified *in vitro* (Fig. 3). Although the different activities showed a variable sensitivity to pH, all enzyme activities were seen to diminish at low pH (approx. 50% activity at pH 5–6).

![Fig. 2. Inhibition of *L. lactis* subsp. *cremoris* MG 1363 by sodium lactate at pH 6·6 (○) and 4·7 (●) on MS10/glucose medium. The maximum specific growth rate (*μ*<sub>max</sub>) was found to be 0·74 h<sup>−1</sup> in MS10 medium at pH 6·6 and in the absence of sodium lactate supplements.](image)
dent of the pH for half the glycolytic genes, namely used. Constant expression profiles were obtained indepen-

between 0

the ratios of standardized expression values were generally

various transcript profiles relative to pH. At a given pH,

obtained at pH 6

? 

cellular concentration was standardized against the value

et al

cellular concentration of each specific messenger (Fontaine

pathway gene transcripts was measured using DNA macro-

pH 6

? 

a growth rate of 0

±

Mean data correspond to three independent values and are shown ±SD. Yxs, biomass yield relative to glucose (glc) con-

sumption; vATP, specific rate of ATP production (vLactate+Vformate+Vacetate); YATP, biomass yield relative to ATP production (μl/vATP); p.m.f., proton motive force.

<table>
<thead>
<tr>
<th>Parameter [unit(s)]</th>
<th>Value of parameter at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>Yxs (g glc mol⁻¹)</td>
<td>9.6±0.6</td>
</tr>
<tr>
<td>vATP [mmol (g cells)⁻¹ h⁻¹]</td>
<td>18.6±0.9</td>
</tr>
<tr>
<td>YATP (g ATP mol⁻¹)</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>ΔpH</td>
<td>0.49±0.10</td>
</tr>
<tr>
<td>pHi</td>
<td>5.7</td>
</tr>
<tr>
<td>ΔΨ (mV)</td>
<td>−86±9</td>
</tr>
<tr>
<td>p.m.f. (mV)</td>
<td>−115±15</td>
</tr>
<tr>
<td>ATP (mM)</td>
<td>3.8±0.7</td>
</tr>
</tbody>
</table>

Transcript profile

The total RNA concentration in the cells was estimated in the four steady-state conditions (Table 4) and was approxi-
mately twofold lower at pH 5.2 and 4.7 compared to pH 6.6. The abundance of glycolytic and fermentative pathway gene transcripts was measured using DNA macro-

arrays (expressed as alkaline phosphatase activity) and corrected by the cellular RNA concentration to obtain the cellular concentration of each specific messenger (Fontaine et al., 2001; Even et al., 2001, 2002). For each transcript, the cellular concentration was standardized against the value obtained at pH 6.6 (Table 5, data in parentheses). The set of experiments was repeated three times to confirm the various transcript profiles relative to pH. At a given pH, the ratios of standardized expression values were generally between 0.8 and 1.2, which did not exceed the value of 1.6 representing the inherent error of the analytical procedures used. Constant expression profiles were obtained independent of the pH for half the glycolytic genes, namely pkf, fbaA, tpi, pgk and enoA, since only small variations of trans-

cript concentration were found compared to the precision threshold value of the method. For the other genes, pgIA, gap and pyk, transcript concentrations did not show appreciable variation in cells grown between pH 6.6 and 5.2, but they did decrease slightly at pH 4.7. In the case of pmg, the slight decrease of transcript concentration was observed earlier in the range of pH values. It is interesting to note that these variations in cellular mRNA content are not apparent if data are examined simply at the level of abundance.

Regulation analysis

An increase in the glycolytic rate under acidic conditions can be achieved by a change in enzyme concentration (hierarchical regulation) and by metabolic regulation [allo-

steric regulation or (co-)substrate saturation]. To quantify the relative importance of both modes of regulation, the approach developed by ter Kuile & Westerhoff (2001) was

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme activity [mmol (g dried cells)⁻¹ h⁻¹] at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>188±8</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>30±3</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate aldolase</td>
<td>138±1</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>2260±200</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>34±6</td>
</tr>
<tr>
<td>3-Phosphoglycerate kinase</td>
<td>52±4</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>145±15</td>
</tr>
<tr>
<td>Enolase</td>
<td>118±1</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>60±2</td>
</tr>
</tbody>
</table>

Table 3. Specific enzyme activities in L. lactis subsp. cremoris MG 1363 during continuous culture on MS10/glucose medium at a growth rate of 0.1 h⁻¹ and different pH values

Mean data correspond to three independent values and are shown ±SD.
used and the hierarchical \( (r_{h,J}) \) and metabolic \( (r_{m,J}) \) regulation coefficients, defined as the following, were estimated for each enzyme.

\[
\rho_{h,J} = \frac{\partial \ln V_i}{\partial \ln c_i} \times \frac{\partial \ln c_i}{\partial \ln J} \approx \frac{\partial \ln c_i}{\ln J},
\]

\[
\rho_{m,J} = \sum X \frac{\partial \ln V_i}{\partial \ln X} \times \frac{\partial \ln X}{\partial \ln J}, \quad \rho_{h,J} + \rho_{m,J} = 1
\]

\( J \) represents the pathway flux, \( c_i \) the concentration of an enzyme through which the flux runs at a rate \( V_i \). \( X \) represents any varying metabolites around the enzyme. The hierarchical regulation coefficient was calculated by the slope of the double logarithmic plot of enzyme concentration towards glycolytic flux (Fig. 4), and coefficients of between 0·4 and 1·0 were obtained (Table 5). The metabolic regulation coefficients calculated by difference \( (1-\rho_{h,J}) \) were in the same range of magnitude (between 0 and 0·6).

To better characterize the effect of acidification on cell growth, the pH was treated separately \( (\rho_{pH,J}) \) and was not included in the metabolic regulation coefficient \( (\rho_{m,J}) \). For each enzyme, this pH regulation coefficient was calculated from the effect of the pH on glycolytic enzyme activity and the observed changes in the pHin, as indicated by the following formula.

\[
\rho_{pH,J} = \frac{\partial \ln V_i}{\partial \ln pHin} \times \frac{\ln pHin}{\partial \ln J}
\]

High negative values for pH regulation coefficients between −0·4 and −3·6 were obtained (Table 5). The sum of the coefficients being equal to 1 \( (\rho_{h,J} + \rho_{pH,J} + \rho_{m,J} = 1) \), very high positive values of between 1·0 and 3·9 were found for the new metabolic coefficients.

The regulation of the enzyme pools was investigated using such a quantitative approach. In growing cells, for which the rate of enzyme degradation can be neglected, the cellular concentration of an enzyme is dependent upon the pool of the corresponding transcript, the translation efficiency \( (k') \) and the growth rate \( (\epsilon_i = k'[mRNA]/\mu) \) (Even et al., 2001). In the particular case of a chemostat culture at a constant
Intracellular concentrations of a transcript normalized using the intracellular concentration at pH 6.6 are shown in parentheses. Transcript abundance is expressed as alkaline phosphatase activity (AP; pixel density min⁻¹) and was calculated using \( \frac{\text{AP[RNA]}}{\text{AP}_{\text{pH e.}}[\text{RNA}]_{\text{pH e.}}} \). Only expressed ratios of \( \geq 1.7 \) or \( \leq 0.6 \) are significant, taking into account the precision of the method (36%).

### Table 4. Transcript abundance and total RNA concentrations during continuous culture of L. lactis subsp. cremoris MG 1363 on MS10/glucose medium at a growth rate of 0.1 h⁻¹ and different pH values

<table>
<thead>
<tr>
<th>Transcript</th>
<th>RNA measurement at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>RNA total (%)</td>
<td></td>
</tr>
<tr>
<td>pgkA</td>
<td>96 (0.5)</td>
</tr>
<tr>
<td>pfk</td>
<td>197 (0.9)</td>
</tr>
<tr>
<td>fhaA</td>
<td>422 (0.8)</td>
</tr>
<tr>
<td>tpi</td>
<td>128 (0.8)</td>
</tr>
<tr>
<td>gap</td>
<td>407 (0.5)</td>
</tr>
<tr>
<td>pgk</td>
<td>226 (0.7)</td>
</tr>
<tr>
<td>pmg</td>
<td>28 (0.3)</td>
</tr>
<tr>
<td>enoA</td>
<td>545 (0.6)</td>
</tr>
<tr>
<td>pyk</td>
<td>233 (0.4)</td>
</tr>
</tbody>
</table>

### Table 5. Quantification of the regulation determinants in L. lactis subsp. cremoris MG 1363 grown in continuous culture on MS10/glucose medium at a growth rate of 0.1 h⁻¹ and different pH values

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Regulation coefficient</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( \rho_{\text{p},1} )</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>0.7</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>0.7</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate aldolase</td>
<td>0.6</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>0.8</td>
</tr>
<tr>
<td>Glyceroldehyde-3-phosphate dehydrogenase</td>
<td>ND*</td>
</tr>
<tr>
<td>3-Phosphoglycerate kinase</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>0.5</td>
</tr>
<tr>
<td>Enolase</td>
<td>0.4</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>ND†</td>
</tr>
</tbody>
</table>

ND, No data.

*No straight line of ln(enzymic activity) versus ln(\( q_0 \)) between 6.6 and 4.7 (see Fig. 4).
†Unknown flux through the enzyme, since the partition between glucose phosphotransferase system and permease has not been characterized.

Dilution rate, and assuming that all the variables are independent, the derivatization of the equation illustrates that the regulation of enzyme level is shared between the transcriptional and translational regulations.

\[
1 = \frac{\text{dlnRNA}}{\text{dln} e_i} + \frac{\text{dln} k^*}{\text{dln} e_i} = \rho_{\text{T},e_i} + \rho_{\text{pH},e_i}
\]

The transcriptional regulation coefficient \( (\rho_{\text{T},e_i}) \) was then calculated for each enzyme, and negative values were obtained for all enzymes except glyceraldehyde-3-phosphate dehydrogenase. When the translational regulation coefficient was calculated \( (\rho_{\text{pH},e_i} = 1 - \rho_{\text{T},e_i}) \), very high positive values were obtained (between 0.7 and 17.5).

### DISCUSSION

**Acid stress damages**

Under acidic conditions, the efficiency of biomass synthesis relative to glucose consumption or ATP production was decreased compared to pH 6.6. This implied that the part of energy dedicated to maintenance by L. lactis was increased at low pH, as described previously for lactic acid bacteria (Kashket, 1987). A fraction of this energy is probably re-routed towards ATPase activity in order to increase the APH under acidic conditions. This enzyme, found to be induced at low pH (Kobayashi et al., 1986; Nannen & Hutkins, 1991b; O’Sullivan & Condon, 1999), is able to extrude protons coupled to ATP consumption and thereby contributes to the maintenance of a favourable cellular pH.

This lower biomass efficiency was related to the pHin decrease, since pHin values fell as low as 5.2 while growth arrest generally occurred for pHin values of between 5.4 and 5.9 (O’Sullivan & Condon, 1997; Mercade et al., 2000), and to organic acid inhibition. Indeed, organic acid inhibition strongly increased at low pH, as illustrated by the growth inhibition profiles induced by sodium lactate at pH 6.6 and 4.7. Inhibition was so pronounced at pH 4.7 that the steady-state was not imposed by carbon limitation but by the maximum lactate accumulation that cells could tolerate while sustaining a growth rate of 0.1 h⁻¹. Indeed, the pulse experiment consisting of the addition of lactate to the fermenter resulted in cell washout until the steady-state lactate concentration was restored. It is interesting to note that the accumulation of glucose at pH 4.7 restores homolactic fermentation, as has been shown under chemostat conditions at high growth rates (Thomas et al., 1979). The regulation of pyruvate formate lyase (PFL) may contribute to this metabolic shift since this enzyme has been identified as a key enzyme under neutral conditions (Melchiorsen et al., 2001). Furthermore, PFL is known to be more sensitive to low pH than lactate dehydrogenase (Asanuma & Hino, 2000). This deviation of metabolism towards a homolactic profile is, however, consistent with
less-intense inhibition by lactate, as compared to formate. Indeed, the lactate inhibition constant \((K_i = 470 \text{ mM})\) was found to be considerably higher than the value previously determined for formate \((K_i = 76 \text{ mM})\) (Loubiere et al., 1997).

**Response to acid stress**

Since acidification provokes pleiotropic effects on cellular physiology [cell membrane damage, inhibition of enzymic activities and transport systems (Hutkins & Nannen, 1993; Poolman et al., 1987a)], the cellular response of *L. lactis* must also be pleiotropic. The increase of \(\Delta pH\) tends to minimize cytoplasmic acidification thus counteracting acid stress damage. This increase of \(\Delta pH\) has been observed for various organic-acid-producing bacteria under acidic conditions (Nannen & Hutkins, 1991a, b; Hutkins & Nannen, 1993; Mercade et al., 2000; Siegumfeldt et al., 2000) and appears to be a common mechanism. A more-global response, never described before, consisting of a significant increase of the glycolytic rate, and thereby the rate of ATP generation, was also observed under acidic conditions. This mechanism appeared as the cellular response to the high-energy requirements linked to acid inhibition. Surprisingly, in these inhibited cells, the intracellular ATP concentration increased when the pH decreased and this is susceptible to favour proton extrusion through the ATPase. Such an increase of the catabolic rate was not observed by O’Sullivan & Condon (1999), though this can probably be explained by the restricted range of growth conditions employed in their study \((\text{pH} \leq 6.25)\). In batch cultures, both the specific rate of glucose consumption and the ATP pool were found to decrease drastically with the progressive acidification of the medium (Cook & Russel, 1994; Even et al., 2002). However, batch cultures in which acidification occurs rapidly may not provoke the same response as chemostat cultures in which long-term responses allow a distinct physiological capacity to form.

When the pH decreased from 5.2 to 4.7, the specific rate of glucose consumption did not increase further. However, an additional metabolic phenomenon was observed since amino acid catabolism, including reactions of deamination, increased significantly as illustrated by the sixfold increase of the specific rate of \(\text{NH}_3\) formation compared to pH 6.6. Furthermore, decarboxylation, generating electrical...
Regulation of the glycolytic rate under acidic conditions

The increased catabolic rate constitutes one of the main cellular responses towards acidification and is widespread over all the range of acidification. An increase in the glycolytic rate can be achieved both by a change in enzyme concentration (hierarchical regulation) and metabolic regulation [allosteric regulation, (co-)substrate saturation]. Intermediary values (between 0.4 and 1) were obtained for hierarchical regulation coefficients indicating that the regulation over the increased glycolytic rate was shared between the modified enzyme concentrations and metabolic regulation of enzyme activity. Furthermore, the regulation is shared fairly equally since similar metabolic regulation coefficients were obtained (between 0 and 0.6). The increase of enzyme activity under acidic conditions is then significant compared to flux variations and participates actively in flux changes. However, when the alternative analysis was undertaken, taking into account the pH effect as a dissociated entity, the metabolic regulation of enzyme activity appeared as the major influence under acidic conditions. Indeed, very high positive values (between 1-0 and 3-9) were obtained for metabolic regulation coefficients. Metabolic regulation, almost completely compensated by the high negative regulation by the pH, enables pathway flux to be attained despite an important decrease of enzyme activity brought about by the diminished pH.In.

The demonstrated hierarchical regulation under acidic conditions prompted us to investigate quantitatively the phenomena regulating the cellular concentrations of glycolytic enzymes in L. lactis. High negative values were obtained for the transcriptional regulation coefficient, illustrating that the enzyme pool generally increased despite the fact that mRNA concentrations remained constant or decreased slightly. Such values indicate that the mechanism by which enzyme concentrations increased could not be attributed to the regulation exerted at the level of transcription. However, when the translational regulation coefficient was calculated, very high positive values were obtained (between 0-7 and 17-5) indicating that translational regulation is preponderant. The translational efficiency (k') increases at low pH (Table 6) and this is presumably associated with an increase in ribosomal activity. As a consequence, a more-rapid rate of enzyme synthesis and, therefore, higher enzyme concentrations were obtained despite the lack of any increase in mRNA concentrations under acidic conditions. Furthermore, this phenomenon was accompanied by a strong decline in the total cellular RNA concentration, also suggesting optimization of the quantity of active ribosomes. It has previously been shown that, at low growth rates, ribosome activity was not maximal (Nierlich, 1978), rendering possible such an optimization of the translation apparatus. The full implication of this increase in translational efficiency will probably become apparent when full-genome arrays coupled to proteome analysis are available, since it is possible that this phenomenon is specific to essential energy-generating pathways, implying selective translational regulation mechanisms.

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REFERENCES


