Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling

Bart Pieterse,^{1,2†} Rob J. Leer,² Frank H. J. Schuren²
and Mariët J. van der Werf^{1,2}

Correspondence

Bart Pieterse

Bart.Pieterse@BDS.nl

¹Wageningen Centre for Food Sciences, Diedenweg 20, 6700 AN, Wageningen, The Netherlands

²TNO Quality of Life, Utrechtseweg 48, 3700 AJ Zeist, The Netherlands

The organic acid lactate is the predominant fermentation product of *Lactobacillus plantarum*. The undissociated form of this organic acid is a strong growth inhibitor for the organism. Different theories have been postulated to explain the inhibitory effects of lactic acid: (i) toxicity arising from the dissipation of the membrane potential, (ii) acidification of the cytosol, or (iii) intracellular anion accumulation. In general, organic acid stresses are complex to study, since their toxicity is highly dependent on their degree of dissociation and thus on the pH. In this study, transcription profiles of *L. plantarum* grown in steady-state cultures that varied in lactate/lactic acid concentration, pH, osmolarity and absolute and relative growth rate, were compared by microarray analysis. By doing so, the differential expression of multiple groups of genes could specifically be attributed to the different aspects of lactic acid stress. A highly coherent group of lactic acid-responsive, cell surface protein-encoding genes was identified, to which no function has previously been assigned. Moreover, a group of genes that showed increased expression in response to the combination of lactic acid and a lower growth rate is expected to be involved in the formation of the alternative fermentation end-products malate, acetate and ethanol. One of these pathways is the phosphoketolase by-pass that is typical for bifidobacteria.

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INTRODUCTION

Lactobacilli are commonly used in fermentation processes for the production of lactate and the conservation of food and feed products. The predominant metabolite biosynthesized during these fermentations is lactate, which is formed as the end-product of glycolysis when pyruvate is reduced to lactate, with NADH as a cofactor, and secreted into the culture medium. The resulting increase in extracellular lactic acid coincides with a decrease of the pH. Lactic acid, together with other organic acids, is known for its inhibitory effect on bacterial growth (Shelef, 1994). Although *Lactobacillus plantarum* is more resistant towards lactic acid than many other micro-organisms, its growth is still strongly inhibited by the elevated concentrations of this organic acid reached during fermentation (Giraud *et al.*, 1991; Russell & Diez-Gonzalez, 1998). The inhibitory effect of organic acids is mainly caused by the undissociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol (Axe & Bailey, 1995;

Hutkins & Nannen, 1993). This process continues until an equilibrium has been reached at which the intra- and extracellular concentrations of the undissociated, membrane-permeable form of the lactic acid molecule are equal. Inside the cell, the lactic acid dissociates in accordance with the Henderson–Hasselbach equation. This implies that the inhibitory effect is larger at a larger ΔpH . It also explains why microbes that can lower their intracellular pH values are more resistant towards organic acids (Cook & Russell, 1994; Diez-Gonzalez & Russell, 1997), as has also been described for *L. plantarum* (McDonald *et al.*, 1990).

Several theories have been postulated to explain the toxic effect of organic acids in more detail. One of these considers organic acids as uncouplers that transport protons towards the inside of the cell, which is a ΔpH -driven process. Eventually, this influx could lead to a complete dissipation of the proton motive force (Axe & Bailey, 1995; Kashket, 1987). A second aspect relates to the deleterious effects of the lower intracellular pH, caused by the influx of lactic acid. However, whereas many organisms aim at maintaining a constant intracellular pH (Padan *et al.*, 1981; Shelef, 1994), most anaerobic fermenting species avoid a large ΔpH by allowing

[†]Present address: BioDetection Systems, Kruislaan 406, 1098 SM Amsterdam, The Netherlands.

for a lower intracellular pH, and as a result increase their tolerance to organic acids (Booth, 1985; Cook & Russell, 1994; Diez-Gonzalez & Russell, 1997). This is also the case for *L. plantarum*, which can grow at intracellular pH values as low as 4.6–4.8 (McDonald *et al.*, 1990). A third factor explaining the inhibitory effect of organic acids is the intracellular accumulation of anions, which could lead to both end-product inhibition and a loss of water activity (Russell, 1992; Russell & Diez-Gonzalez, 1998). For lactic acid bacteria, end-product inhibition by lactic acid could result in a disturbance of the regeneration of the cofactor NAD^+ , especially under anaerobic conditions in which the cell does not have the possibility of NAD^+ regeneration by NADH oxidase.

The recent sequencing of the genome of *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003) revealed several genes for which homologues in other prokaryotes have been found to play a role in pH control or in the maintenance of the proton motive force: a proton-translocating F_1F_0 -ATPase (Cotter & Hill, 2003); several sodium-proton antiporters (Cotter *et al.*, 2001); amino acid decarboxylases that use an intracellular hydrogen ion for the decarboxylation of an imported amino acid (Azcarate-Peril *et al.*, 2004; Cotter & Hill, 2003; van de Guchte *et al.*, 2002); and the genes involved in malolactic fermentation, during which extracellular malate is imported and decarboxylated. The lactate that is produced is secreted via an electrogenic uniport, which results in a membrane potential that can be applied to the generation of ATP (Olsen *et al.*, 1991).

The genomic sequence of *L. plantarum* gives insight into potential responses of the cells to counteract the anticipated deleterious aspects of extracellular lactic acid accumulation. Nevertheless, the genomic data give little clue to the exact effects of lactic acid and the response of the cells towards this stress. Functional genomics techniques offer good opportunities for gaining a better understanding of the physiological state and the specific response of the cell. In recent years, the acid stress response of several prokaryotes has been studied with both proteomics and transcriptomics approaches (Ang *et al.*, 2001; Fisher *et al.*, 2002; Giard *et al.*, 2001; Len *et al.*, 2004; Lim *et al.*, 2000; Merrell *et al.*, 2003; Stancik *et al.*, 2002; Wen *et al.*, 2003; Wilkins *et al.*, 2002). A few reports describe the use of these approaches to study the organic acid stress response caused by lactate (Hartke *et al.*, 1996), acetate (e.g. Arnold *et al.*, 2001; Kirkpatrick *et al.*, 2001; Polen *et al.*, 2003), propionate (Polen *et al.*, 2003) and formate (Kirkpatrick *et al.*, 2001). None of these studies, however, allows for discrimination between the effects of the undissociated and the dissociated forms of lactic acid. Moreover, the negative effect that the organic acid could have on the growth rate of the organism, and the secondary effects on gene expression resulting thereby, are usually neglected.

The aim of this study was to obtain a better insight into the effect of lactic acid on *L. plantarum*, and to obtain a genome-wide view of the transcriptional response towards this stress

factor. To this end, an experimental design was developed that allows discrimination of the effects of pH, lactic acid in its dissociated and undissociated forms, and the secondary effect of a diminished maximum growth rate.

METHODS

Micro-organism. The micro-organism used for these experiments was *L. plantarum* WCFS1, a single-colony isolate from *L. plantarum* NCIMB8826, which is maintained at NIZO food research in Ede, The Netherlands. The complete genome sequence of this organism is available (Kleerebezem *et al.*, 2003; GenBank, AL935263).

Cultivation and cell harvesting. Glucose-limited continuous cultivation of *L. plantarum* WCFS1 was performed in duplicate on 25% MRS medium (13.75 g l^{-1} , Difco) with additional sodium chloride or sodium lactate (as specified in Table 1), at 37°C in an Applikon bioreactor with a working volume of 1 l. Glucose limitation was confirmed by the fact that the addition of a concentrated glucose solution to the steady-state culture resulted in an increased cell density. A constant pH was maintained (pH 6.0 or 4.8) by automatic titration with 10 M sodium hydroxide. The culture was kept anoxic by a continuous overlay with nitrogen gas. The dilution rate was set at 0.3 h^{-1} or 0.05 h^{-1} .

Samples were taken from the steady-state cultures and immediately quenched in a -45°C methanol-based buffer, as previously described (Pieterse *et al.*, 2005). Cell pellets were stored at -80°C until use.

RNA isolation. RNA was isolated from the cells according to the Macaloid/phenol-based protocol, as previously described (Pieterse *et al.*, 2005). RNA purity and concentrations were determined both spectrophotometrically and on agarose gel. The RNA isolates were checked for residual RNase activity by comparing samples that were incubated for 1 h at 42°C with the initial material on an agarose gel.

Array design. The microarray used is a clone-based array, containing 3714 identified unique fragments from the genomic *L. plantarum* WCFS1 library in pBlueScript SK+ (Kleerebezem *et al.*, 2003). The mean size of the fragments is 1.2 kb, with a standard deviation of 0.3 kb. The microarray contains approximately 80% of the *L. plantarum* WCFS1 genome (Pieterse *et al.*, 2005).

Fluorescent labelling and hybridization. Differential transcript levels were determined by two-colour fluorescent hybridizations of the corresponding cDNAs on the *L. plantarum* clone array. The RNA samples were labelled by *in vitro* reversed transcription with either Cy5- or Cy3-labelled dUTP, using random hexamer primers. Labelling, hybridization and washing were performed as previously described (Pieterse *et al.*, 2005). The fluorescent labels were swapped for the labelling of the biological duplicates in order to avoid false positives due to dye-specific effects.

Image analysis. The fluorescent signals from the two different labels on the hybridized arrays were quantified with a ScanArray Express scanner (Packard Bioscience) and Imagen 4.2 software (BioDiscovery, Inc.). Spots for which the difference between the mean signal of the spot and the mean signal of the background was smaller than two times the background standard deviation were excluded from further analysis. Spots for which the signal in one or both of the channels exceeded the detection limit of the scanner were also excluded. After removal of the empty spots and the spots for which the signal exceeded the detection limit of the scanner, 2787 spots (75%) remained for further analysis.

Normalization. Within-slide, intensity-dependent normalizations were performed with the scatter plot smoother LOWESS from the

software program Datafit (Oakdale Engineering). The user-defined fraction of data used for smoothing at each point was set at 20% for all slides.

Significance analysis. Prior to significance analysis, a data transformation was applied to the normalized ratios in order to obtain a distribution around zero that approached the normal distribution (Pieterse *et al.*, 2005). Significance analysis was performed by one-way ANOVA. Subsequently, a Tukey HSD test was performed to determine whether a significant differential expression level (99% confidence interval) was observed under a specific condition. If genes or predicted operons were present on multiple spots on the array, these spots were considered as replicates in the significance analysis.

By comparison of overlapping regions of the genomic fragments on the array, we were able to predict which of the specific genes present in the clones were the ones affected.

Hierarchical clustering. For the hierarchical clustering and visualization of the results, the programs CLUSTER and TREEVIEW were applied (Eisen *et al.*, 1998). Only those genes or operons were included that fell within the 99% confidence interval of the Tukey HSD test for at least one of the six conditions. Average linkage clustering was performed on the natural logarithm of the expression ratios of the genes or operons, based on all replicate spots. The outcome of the clustering was influenced by assigning weight values to the different experiments. For the experiments in which the effect of lactate was studied, a weight value of 1 was applied. The dataset in which the effect of the lower pH value was studied was assigned a weight value of 0.75, the 800 mM sodium chloride dataset 0.5 and the 300 mM sodium chloride dataset 0.25.

In silico analysis of cell surface proteins. The intracellular, extracellular and transmembrane regions of proteins that were annotated as cell surface proteins were predicted using a hidden Markov model-based tool (<http://www.cbs.dtu.dk/services/TMHMM>; Krogh *et al.*, 2001). Multiple protein alignments (gap open penalty of 10; gap extension penalty of 1; GONNET-matrix), and pI predictions were performed in DNAMAN version 5.2.9. (Lynnon BioSoft). The motif LPQTxE is the *L. plantarum* orthologue of the well-known cell-wall-anchoring LPxTG pentapeptide (Fischetti *et al.*, 1990; Kleerebezem *et al.*, 2003).

Scanning electron microscopy. Nucleopore polycarbonate membranes (Costar) with 1 µm pores were coated with poly-L-lysine (30 min in 0.01% poly-L-lysine, 0.1 M Tris/HCl buffer). For scanning electron microscopy, several drops of bacteria from duplicate

steady-state cultures were transferred to the membrane immediately upon harvesting, and stored for 5 min at 100% humidity. Fixation was achieved by 30 min incubation in the bacterial growth medium with additional 3% glutaraldehyde. The membranes were subsequently rinsed three times in water for 10 min. Dehydration was performed by immersing in a series of ethanol washes (30, 50, 70, 90, and three times 100%). Membranes were critical point dried in CO₂ (Balzers CPD 020, Balzers Union). The dry membranes were mounted on the sample holders by carbon adhesive tabs (Electron Microscopy Sciences). Sample holders were put inside the preparation chamber (CT 1500 HF, Oxford Instruments) attached to the microscope. Samples were sputter coated with 5 nm platinum and analysed in a field emission scanning electron microscope (JEOL 6300F) at 5 kV. Images were recorded digitally, and photo processing was done with Adobe PhotoShop 5.5.

RESULTS AND DISCUSSION

Experimental design

The discrimination between the effects on bacterial physiology of the dissociated and undissociated form of organic acids has been the subject of many studies (e.g. Kashket, 1987; Salmond *et al.*, 1984). However, so far, these studies have not been extended to the gene expression level. In this study, an experimental design was applied that enabled discrimination between the effects of lactic acid in its dissociated and undissociated form, pH, water activity, and absolute and relative growth rate (Table 1, Fig. 1). Cells were grown in steady-state cultures, which allows for a reliable view of the long-term effects of the organic acid.

In order to distinguish between the effects of the undissociated (uncharged) and dissociated (charged) form of the molecule, fermentations with 300 mM additional sodium lactate (the lactate concentration resulting from the fermented glucose was approximately 50 mM) were performed at two pH values, 6.0 and 4.8. In this way, the amount of undissociated lactic acid could be varied (2.1 versus 30.7 mM additional undissociated lactic acid), while maintaining an equal amount of initial total lactate. The effect of the additional sodium lactate at pH 6.0 will be referred to as the lactate effect, while that at pH 4.8 will be referred to as

Table 1. Culture conditions of the fermentations with *L. plantarum* for which transcription profiles were compared by microarray analysis

abs. μ , absolute growth rate; rel. μ , relative growth rate; $\mu_{\max}$, maximum growth rate; d , dilution rate. The units of growth rate are h^{-1} ; the units of dilution rate are h^{-1} .

Code	Effect	Stress condition					Reference condition				
		pH	Addition	$\mu_{\max}$	d	rel. μ	pH	Addition	$\mu_{\max}$	d	rel. μ
A	Mild osmotic stress	6.0	300 mM NaCl	0.6	0.3	0.5	6.0	No addition	0.6	0.3	0.5
B	Osmotic stress	6.0	800 mM NaCl	0.4	0.3	0.8	6.0	No addition	0.6	0.3	0.5
C	Low pH	4.8	300 mM NaCl	0.5	0.3	0.6	6.0	300 mM NaCl	0.6	0.3	0.5
D	Lactate	6.0	300 mM Na-lactate	0.6	0.3	0.5	6.0	300 mM NaCl	0.6	0.3	0.5
E	Lactic acid/higher rel. μ	4.8	300 mM Na-lactate	0.06	0.05	0.8	4.8	300 mM NaCl	0.5	0.05	0.1
F	Lactic acid/lower abs. μ	4.8	300 mM Na-lactate	0.06	0.05	0.8	4.8	300 mM NaCl	0.5	0.3	0.6

Fig. 1. Schematic representation of the experimental design that was applied for discriminating between the effects of lactate, lactic acid, pH and osmotic stress on gene expression of *L. plantarum* WCFS1. Each bioreactor in the scheme represents a steady-state fermentation in duplicate. The connectors between the bioreactors represent the hybridizations as they were performed. The letter codes correspond with those in Table 1.

the lactic acid effect. The dilution rate of the cultures with a pH of 6.0 was 0.3 h⁻¹, and that of the cultures at pH 4.8, 0.05 h⁻¹. The transcription profiles from these cultures were compared with those of cultures to which an equal concentration of sodium chloride, which causes an equal reduction of the water activity, was added (Houtsma *et al.*, 1993). All other parameters were constant (Table 1, rows D and E).

The effect of extracellular pH on gene expression was studied by comparing mRNA levels from *L. plantarum* cultured at pH 4.8 with those of *L. plantarum* cultured at pH 6.0 (Table 1, row C).

As the presence of lactic acid also influences the growth rate of the organism, changes in gene expression are expected that are not caused by the primary effect of the sodium lactate, but by the secondary effect of an altered absolute or relative growth rate. In this study, the effect of a different absolute growth rate was avoided by using identical dilution rates for the cultures for which the transcription profiles were compared. In order to determine whether relative growth rate (i.e. the ratio between the set growth rate and the maximum growth rate under the given conditions in a batch culture) also affected gene expression, the transcription profile of the culture with sodium lactate at pH 4.8 and a dilution rate of 0.05 h⁻¹ was also compared with that of the culture with 300 mM sodium chloride at pH 4.8 and a dilution rate of 0.3 h⁻¹. The relative growth rates of these cultures were alike (Table 1, row F).

Furthermore, transcriptome datasets from two independent duplicate cultures in which the effect of additional sodium chloride (0 mM versus 300 mM/0 mM versus 800 mM)

was studied, were generated (Table 1, rows A and B). These were added to study whether osmotic stress mechanisms are initiated in the presence of lactic acid.

Hierarchical cluster analysis

Hierarchical clustering was performed in order to group genes or operons that showed similar expression patterns under the different experimental conditions (Fig. 2).

The hierarchical cluster clearly showed that the number of genes that were affected by the addition of 300 mM sodium chloride, a lower pH and lactate at a pH of 6, is rather

Fig. 2. Hierarchical cluster of the normalized, log-transformed data of the mean gene expression for all genes for which a significant differential expression (99% confidence interval of the Tukey HSD test) was observed for at least one of the conditions. Red, increased expression; green, decreased expression; black, unchanged expression. The groups of genes marked in the numbered boxes are listed in Tables 2–7.

limited. The limited number of genes that were affected by a lower pH alone is not surprising in view of the limited effect that this pH difference has on growth rate and biomass yield (Giraud *et al.*, 1991). However, according to McDonald *et al.* (1990), such a decrease in extracellular pH could coincide with a decrease of the intracellular pH of almost 1 pH unit. Apparently, this decrease in intracellular pH is not itself a strong trigger for gene regulation. The combined facts that a relatively low number of genes were differentially expressed in response to lower pH alone and to additional sodium lactate at a pH of 6.0, support the theory that the effect of lactic acid is mainly caused by the undissociated form of the molecule.

Striking differences occurred in the two datasets that represent the effect of lactic acid, but vary in growth rate/relative growth rate. In order to determine lactic acid-specific effects, we concentrated on the genes that are marked with boxes 1 and 2 in Fig. 2. The corresponding genes and their expression data are listed in Tables 2–7.

Identification of genes with a specifically increased expression in response to lactic acid

The focus of this research was on the identification of genes that showed an increased expression in response to lactic acid, regardless of absolute and relative growth rate. These

features are shown by the 18 genes or operons in group 4 (Fig. 2, Table 2). Several of these also showed an increased expression in response to other environmental differences (especially the presence of 800 mM NaCl).

Probably the most striking feature from this group of genes was the multiple cell surface protein-encoding genes that were found. Analysis of the corresponding regions of these genes on the *L. plantarum* genome indicated that three operons encoding cell surface proteins could be identified, not all the genes of which were represented in our datasets. The corresponding proteins showed low similarity to other proteins in database searches. Prediction of transmembrane helices, cell wall anchors, expected pI values and amino acid lengths from the complete sets of genes revealed a structural homology between these three operons (Fig. 3). This 'structural conservation' might indicate that the proteins within a single operon interact. Multiple sequence alignment of the amino acid sequences from the corresponding genes showed a limited number of highly conserved regions (data not shown). The array data indicated that, under inducing conditions, the absolute expression level of the lp₃₆₇₉–lp₃₆₇₆ operon was among the highest transcript levels measured. This would suggest that the corresponding proteins are abundantly present on the cell surface. Indeed, scanning electron microscopy revealed striking morphological differences between the stressed and non-stressed cells

Table 2. Genes/operons from group 4 of the hierarchical cluster (Fig. 2)

This group is characterized by the increased expression of genes/operons in response to lactic acid. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95% confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
2109	Excinuclease, subunit C	1.0	1.6	1.3	1.0	1.4	1.4
0027–0028	β -Phosphoglucomutase; glycosylhydrolase	1.0	3.1	1.0	1.3	2.1	2.7
3578	Catalase	1.9	3.6	1.7	1.5	3.1	3.0
0841–0843	Exopolyphosphatase; polyphosphate kinase; exopolyphosphatase	1.4	1.8	1.2	1.1	1.6	1.5
1396	Putative transcriptional regulator	1.0	1.9	0.9	0.8	1.8	2.1
0313	NADH dehydrogenase	1.4	1.6	1.3	0.8	2.6	2.7
3128	Dpr-like protein	2.5	3.1	1.1	0.7	2.7	3.5
3127	Cell surface protein	1.7	2.2	1.3	0.8	2.0	3.2
0965	Transcriptional regulator	0.7	1.9	1.3	0.9	2.5	2.9
2173–2174	Extracellular protein; cell surface protein	0.7	0.9	1.2	1.1	1.6	1.6
3262–3263	Squalene synthase; phytoene synthase	1.0	1.2	1.2	1.3	2.9	2.5
2975–2977	Integral membrane protein; cell surface protein; cell surface protein	0.7	1.2	2.1	1.3	2.6	2.7
1269	Clp protease, ATP-binding subunit clpE	0.8	1.0	1.4	1.1	1.7	1.8
3678–3679	Cell surface protein; cell surface protein	1.0	2.0	4.0	1.2	14.2	12.3
0946	Cell surface protein	0.9	1.1	1.4	1.1	2.2	2.6
1785	Phosphoglycerate mutase	1.1	1.0	1.2	1.0	1.4	1.6
1697	Extracellular protein	1.1	1.1	1.2	1.0	3.2	3.8
3092	Succinate-semialdehyde dehydrogenase	1.2	1.1	1.2	1.1	1.7	1.4

Fig. 3. Diagrammatic representation of three cell surface protein operons that showed increased expression upon continuous lactic acid stress. The proteins were vertically aligned in correspondence with the homology between the operons. The size of the arrows is proportional to the length of the proteins in amino acids. Predicted intracellular regions are depicted in black, predicted transmembrane regions in grey, and predicted extracellular regions in white. The ORF number is shown above the arrows, and the pI values within the arrows. The cell wall anchor motif LPQTxE is shown by a diamond symbol.

(Fig. 4): whereas the cells that were not subjected to lactic acid stress showed a smooth appearance, the stressed cells had a remarkably rough surface. These structures were mainly situated on the longitudinal sides of the cells. It is tempting to speculate that these cell surface proteins may have a structural, physical function in combating lactic acid stress. To our current knowledge, a specific role for structural cell surface proteins in the organic acid stress response has not been implicated before.

Other genes that showed a growth-rate-independent lactic acid response are associated with various stress responses in prokaryotes, i.e. the genes encoding Clp protease, an excinuclease (DNA damage), catalase (peroxide stress), and the Dpr-like protein (peroxide stress) (Hartke *et al.*, 1996; Price *et al.*, 2001; Quivey *et al.*, 1995; Yamamoto *et al.*, 2000). Increased expression was also observed for these genes in cells cultured in the presence of 0.8 M NaCl. These results suggest that lactic acid stress in *L. plantarum* WCFS1 also induces a more general stress response. An overlap between the stimulon for lactic acid and those for peroxide and UV radiation has also been reported for *Lactococcus lactis* (Hartke *et al.*, 1996).

Two other genes that may serve a specific role in the counteracting of lactic acid stress are squalene synthase and phytoene synthase, which are involved in the biosynthesis of sterols. Sterols can increase the rigidity of the membrane

(van der Rest *et al.*, 1995), which could possibly limit the influx of lactic acid.

Moreover, two predicted regulators were represented in the group of genes that showed increased expression in response to lactic acid. These are especially interesting because of their potential role in the regulation of an organic acid stress response.

Identification of genes with a specifically decreased expression in response to lactic acid

Group 2 (Fig. 2, Table 3) contains 10 genes or operons that showed a decreased expression in response to lactic acid regardless of absolute and relative growth rate, several of which also showed a decreased expression in response to a lower pH alone and to lactate at pH 6.0. The putative functions of these genes vary widely.

Effect of lactic acid on the expression of genes that are known for their role in (in)organic acid response

In addition to the genes that were found to show differential expression in response to lactic acid, we studied the expression of a number of genes that have been associated with the prokaryotic (organic) acid response in earlier studies.

Fig. 4. Scanning electron microscopy images from *L. plantarum* WCFS1 cells that were cultured in steady-state cultures at pH 4.8 in the absence (–) or presence (+) of 300 mM additional sodium lactate.

Table 3. Genes/operons from group 2 of the hierarchical cluster (Fig. 2)

This group is characterized by the decreased expression of genes/operons in response to lactic acid. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95 % confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
0124	Cation transporting P-type ATPase	1.0	1.4	0.5	0.4	0.8	0.5
1799	Hypothetical protein	1.2	1.0	0.6	0.7	0.7	0.8
2929–2930	Hypothetical protein; hypothetical protein	1.1	1.0	0.8	0.5	0.4	0.6
2931–2932	Anaerobic ribonucleotide reductase activator protein; anaerobic ribonucleoside-triphosphate reductase	1.3	1.3	0.8	0.7	0.5	0.8
2664–2665	Hypothetical protein; response regulator	1.2	1.3	0.7	0.8	0.6	0.8
2872–2874	Hypothetical protein; alcohol dehydrogenase; oxidoreductase	1.6	1.8	0.5	0.4	0.4	0.6
2325–2326	Thiamine biosynthesis protein; cysteine desulfurase	1.2	0.9	0.8	0.7	0.6	0.9
2142–2143	Glycosyltransferase; hypothetical protein	1.3	1.3	0.7	0.8	0.5	0.5
1568	Penicillin-binding protein	0.9	0.9	0.8	0.9	0.5	0.6
1317–1319	Integral membrane protein; pseudouridylate synthase	0.9	0.9	0.8	0.9	0.7	0.7

Although the role of the F_1F_0 -ATPase in acid adaptation has been clearly demonstrated in studies with both a mutant strain and a strain with an increased F_1F_0 -ATPase activity (Drici-Cachon *et al.*, 1996; van de Guchte *et al.*, 2002), no differential expression of these genes could be observed in this study. It should be noted that these effects were observed at low pH values in the presence of inorganic acids. It is doubtful whether this system plays a significant role in the stress attributed to lactic acid. The amount of energy that this system would require when the cell has to cope with a massive influx of lactate could ultimately lead to a cessation of growth (Bond & Russell, 1996).

If the uncoupling effect is the main factor responsible for lactic acid stress, one would expect major problems in proton motive force-dependent transport processes, whereas the results that are presented in this paper do not indicate a lactic acid-specific adaptation of the expression of genes encoding transport proteins. Furthermore, no increase of lactic acid-specific expression was observed for genes that could play a role in restoring the membrane potential, such as cation transporters and the malolactic fermentation genes. Remarkably enough, a cation transporter (lp_0124) that did respond to lactic acid showed decreased expression (Table 3).

Metabolic rerouting under the combined effects of lactic acid and a lower absolute growth rate

Groups 5 and 3 (Fig. 3, Tables 4 and 5) contain genes and operons that were differently expressed in cells grown in the

presence of lactic acid and with a lower absolute growth rate. Within these clusters, several genes involved in energy metabolism showed a differential expression, which could indicate a shift towards alternative fermentation pathways. The complete dataset was studied in more detail to determine whether other genes in these pathways showed similar trends. An overview of the expression of these genes and the metabolic routes in which they are involved is presented in Table 8 and Fig. 5.

Genes encoding transketolase and transaldolase (lp_3538 and lp_3539), both involved in the pentose phosphate cycle, showed an increased expression (Table 8). Furthermore, an increase of approximately fivefold was observed for the phosphoketolase encoded by lp_3551 (90 % confidence interval; Table 8). The increased expression of both transaldolase and phosphoketolase suggests a shift towards the phosphoketolase bypass, as is typically performed by bifidobacteria (Kandler, 1983). In this pathway, the phosphoketolase uses both xylulose 5-phosphate and fructose 6-phosphate as substrates. Homology searches also suggest that the *L. plantarum* phosphoketolase can convert both substrates (results not shown). As far as we are aware, use of the phosphoketolase bypass has not previously been described for lactobacilli. It is generally suggested that phosphoketolase only serves a role in the heterolactic fermentation pathway, where it converts xylulose 5-phosphate into acetyl-phosphate and glyceraldehyde 3-phosphate (Kandler, 1983).

Table 4. Genes/operons from group 5 of the hierarchical cluster (Fig. 2)

This group is characterized by the increased expression of genes/operons under the combination of lactic acid and a lower absolute growth rate. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95 % confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
0898 or 0899	GroEL suppressor protein SugE/hypothetical protein	0.9	0.8	1.4	1.0	0.9	3.0
3470	Transcription regulator of β -galactosidase gene	1.0	1.2	1.6	1.2	1.3	2.1
1303a	Cell surface SD repeat protein	0.8	1.0	1.5	1.2	1.5	2.3
0779–0780	Nucleotide kinase (putative); hypothetical protein	0.9	1.7	1.5	1.2	1.2	2.5
2668	Small heat-shock protein	0.9	1.1	1.3	1.1	1.3	5.4
1155–1156	Cellobiose PTS, EIIC; cell surface hydrolase (putative)	0.8	1.0	1.2	1.1	1.1	1.6
3658–3660	Ribose uptake and conversion, rbsU; rbsD; rbsK3	1.3	0.8	1.1	1.7	1.6	2.9
3416	Putative transcription regulator	1.6	2.0	0.7	0.9	1.5	6.0
2596	Formate acetyltransferase activating enzyme	1.4	1.8	0.8	0.9	1.7	3.3
3673 or 3675	Cysteine aminopeptidase/signal peptidase I	1.1	1.0	1.0	1.0	1.3	1.7
3207	Aminotransferase	0.5	0.6	1.2	1.3	1.5	1.5
0800	Cell surface protein	0.7	0.6	1.5	1.3	2.0	1.6
3545–3548	L-Iditol 2-dehydrogenase; galactitol PTS, pts32C; pts32B; pts32A	0.4	0.2	1.0	3.9	1.5	6.4
0472–0473	Putative integral membrane protein; hypothetical protein	0.6	0.5	1.1	1.5	1.2	1.4
2570	Aspartate-semialdehyde dehydrogenase	0.8	0.5	1.2	1.1	1.2	1.5
3500–3501	Oxidoreductase; hypothetical protein	0.9	0.6	0.9	1.2	1.1	1.4
2643	Lipoate protein ligase	0.9	0.3	0.7	1.1	1.2	1.9
3418	Phosphoenolpyruvate carboxykinase	1.0	0.2	0.6	1.3	0.8	3.0
1912	Phosphoenolpyruvate synthase	0.5	0.2	0.5	1.6	0.7	7.0
3554–3557	Arabinose uptake and conversion, araD; araD; araB; araT	1.1	0.6	0.6	1.0	1.2	2.7
3520–3524	pts32A; transcriptional regulator; pts32BC; glucokinase regulatory protein; hypothetical protein	1.3	0.6	0.7	1.0	1.0	1.9
0498	Fucose transport protein	0.9	0.3	0.5	0.8	1.1	2.5
3538–3539	Transketolase; transaldolase	1.0	0.7	1.0	1.0	1.2	2.3
3540–3542	Transport protein; hypothetical protein; hypothetical protein	1.1	0.7	0.8	0.8	1.2	2.4
3563–3567	Sorbitol uptake and conversion, pts38BC; pts38C; srlM1; srlR2	1.6	0.8	0.8	0.9	1.1	1.9
1763	Glycosyltransferase	1.0	0.7	1.1	1.0	1.3	1.6
0758	Hypothetical protein	0.9	0.6	0.8	1.1	1.5	2.8
1468–1473	ABC transporter, ATP-binding protein; ABC transporter component (putative); cysteine desulfurase; NifU-like protein; ABC transporter component, iron regulated (putative); iron chelatin ABC transporter, substrate binding protein (putative)	0.8	0.7	0.8	1.2	1.5	1.6

The genes encoding phosphoenolpyruvate synthase (lp_1912) and phosphoenolpyruvate carboxykinase (lp_3418), responsible for the bypass from pyruvate to oxaloacetate via

phosphoenolpyruvate, both showed an increased expression (Table 8). A subsequent conversion to malate by malate dehydrogenase would lead to NAD^+ regeneration. The flux

Table 5. Genes/operons from group 3 of the hierarchical cluster (Fig. 2)

This group is characterized by the decreased expression of genes/operons under the combination of lactic acid and a lower absolute growth rate. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95 % confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
2992	Manganese transport protein	1.1	0.4	7.0	4.9	0.7	0.2
0230–0233	Mannitol uptake and conversion, mtlA; mtlR; mtlF; mtlD	0.9	0.5	1.2	1.0	0.9	0.7
0355	Putative oxidoreductase	0.8	0.4	1.4	1.3	0.8	0.6
0197	Cell surface protein	0.9	0.4	1.4	1.2	0.7	0.7
1497–1500	Nitrate reductase, narG; narH; narJ; narI	0.7	0.5	1.4	1.4	0.8	0.7
2033–2038	Aromatic amino acid biosynthesis, aroI; tyrA; aroE; hypothetical protein; aroF	0.8	0.5	1.1	1.6	0.8	0.5
1084–1086	Aromatic amino acid biosynthesis, aroD1; aroA; aroB	0.8	0.5	1.2	1.6	0.9	0.5
2082 or 2083 or 2084	Hypothetical proteins	0.6	0.4	1.1	1.3	0.8	0.5
3359–3360	Integral membrane protein; integral membrane protein	0.5	0.4	1.8	1.6	1.1	0.6
2839	Hypothetical protein	1.0	1.2	1.4	1.0	0.8	0.5
1941	NADH oxidase	1.0	1.1	1.2	1.0	0.8	0.6
1008	Lysine transport protein	0.5	0.9	1.2	1.2	0.6	0.4
2136	Pyruvate carboxylase	0.8	0.9	1.1	1.0	0.7	0.6
3184–3185	Integral membrane protein; branched-chain amino acid transport protein	0.8	0.7	1.2	1.2	0.5	0.4
1148–1149	Glutamyl-tRNA amidotransferase, subunit A; subunit B	0.9	0.9	1.1	1.2	0.7	0.6
2761–2765	O-Acetyltransferase; ADP-ribose pyrophosphatase; hypothetical protein; short-chain dehydrogenase/oxidoreductase; hypothetical protein	1.0	1.0	0.9	0.8	0.8	0.6
0977	UDP-N-acetylmuramoylalanyl-D-glutamate-2, 6-diaminopimelate ligase	1.0	1.0	1.0	0.9	0.9	0.5
0881–0883	Glutamine ABC transporter, glnH1; glnM; glnP	1.6	0.9	1.0	0.9	0.8	0.3
0254–0256	Cysteine biosynthesis, cysE; metC1; cysK	1.2	0.6	0.8	1.1	0.4	0.2
0190–0192	Hypothetical protein; Na ⁺ /H ⁺ -antiporter	0.7	0.8	1.3	0.9	0.9	0.5
3042	ABC transporter, ATP-binding and permease protein	0.8	0.8	0.9	0.9	1.2	0.4
0113–0116	Thiamine biosynthesis, thiM; thiD; thiE; codB	0.6	0.6	1.1	1.2	1.1	0.4
0571–0572	Threonine biosynthesis, hom2; thrB	0.9	0.7	0.8	1.0	1.0	0.5
3334	Adenine deaminase	0.8	0.2	0.8	0.9	0.7	0.1
2290	Integral membrane protein	0.8	0.8	1.0	1.0	0.8	0.5
0217–0219	ABC transporter, permease protein; ATP binding domain; putative subunit	0.6	0.4	1.2	0.9	0.7	0.2
3085	Asparagine synthase	0.9	0.4	0.9	1.0	1.1	0.5
1262–1265	Oligopeptide ABC transporter, oppC; oppD; oppE; oppF	1.0	0.6	0.9	1.2	1.0	0.5
1164–1165	Cellobiose PTS EIIC fragment; hypothetical protein	0.7	0.5	1.1	1.0	0.9	0.6
0848	Permease	1.2	0.3	0.9	0.7	0.9	0.2
3270	Adenylosuccinate synthase	1.3	0.2	1.1	0.7	2.0	0.1
1729–1731	Maltose metabolism, sugar transporter; map3; galM2	1.0	0.8	1.1	0.9	1.2	0.6
2719–2729	Purine biosynthesis cluster	0.8	0.2	1.3	1.0	2.1	0.2

Table 6. Genes/operons from group 5 of the hierarchical cluster (Fig. 2)

This group is characterized by the increased expression of genes/operons under the combination of lactic acid and a higher relative growth rate. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95 % confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
3267	Dihydrolipoamide dehydrogenase	0.8	1.0	1.3	1.2	2.2	0.5
2277	Alanine-tRNA ligase	0.8	0.8	1.2	1.2	1.6	0.6
2187	Isoleucine-tRNA ligase	0.9	0.8	1.3	1.2	1.7	0.5
3059	Cell surface protein	1.1	0.8	1.5	1.1	2.1	0.7
1980–1981	Aspartate-tRNA ligase; histidine-tRNA ligase	1.0	0.9	1.1	1.3	1.7	0.8
1316	Leucine-tRNA ligase	0.9	1.1	1.3	1.2	1.8	0.8
0793	Hypothetical protein	0.8	1.5	1.8	1.2	1.3	1.0
3412–3414	Extracellular protein; cell surface protein; cell surface protein	1.5	1.4	2.6	1.2	1.9	0.8
0520	ATP-dependent RNA helicase	1.0	0.8	1.4	1.0	1.6	0.9
1643–1645	Mucus binding protein; hypothetical protein	0.8	0.6	2.5	1.8	2.5	0.9
2322	Valine-tRNA ligase	0.9	1.1	1.6	1.2	1.8	1.3
1685	Putative transcriptional regulator	1.2	1.2	1.4	1.4	2.6	1.1
1446–1450	Hypothetical protein; cell surface protein; cell surface protein; hypothetical protein; hypothetical protein	0.9	1.2	1.2	1.5	2.0	1.3
3174	Cyclopropane-fatty-acyl-phospholipid synthase	1.3	1.5	1.2	1.1	2.0	1.2
1673–1681	Fatty acid biosynthesis cluster, fabD; fabG1; fabF; accB2; fabZ	1.4	1.1	0.9	0.9	18.5	1.2
0315–0319	Spermidine/putrescine ABC transporter, potA; potC; potB; potA	1.0	0.9	1.0	1.0	1.8	1.1
1021	DNA-directed RNA polymerase, β subunit	0.7	0.8	1.2	0.9	2.1	1.3
0262–0265	Trehalose uptake and conversion, treR; treA; pts4ABC; pts5ABC	1.3	0.8	1.3	1.0	1.8	1.3
2502	Glucose-6-phosphate isomerase	0.9	0.6	1.2	0.9	1.7	1.1

through this pathway seems to be further stimulated by the down-regulation of the pyruvate carboxylase (lp_2136) and pyruvate kinase genes (lp_1897; statistically non-significant) under the same conditions (Table 8).

The increased expression of lp_2596, the formate acetyl transferase activating enzyme (Table 8), suggests the up-regulation of the formate acetyl transferase gene, responsible for the conversion of pyruvate into formate and acetyl-CoA. Indeed, an increase in expression (90 % confidence interval) of this gene (lp_2598) could be observed (Table 8). A subsequent conversion step in this pathway is that of acetyl-CoA to acetaldehyde and ethanol, during which two NAD^+ molecules are regenerated. This step could be performed by the bifunctional alcohol dehydrogenase/acetaldehyde dehydrogenase (lp_3662), for which a statistically non-significant increase in expression of 150 % was observed (Table 8). Alternatively, acetyl-CoA can be converted into acetyl-phosphate and subsequently acetate by phosphotransacetylase and acetate kinase, respectively. However, no increased expression was observed for the corresponding genes.

The suggested reroutings make sense in view of the potential effects of lactic acid accumulation on the cell. Conversion of

pyruvate into lactic acid is the predominant mechanism for *L. plantarum* to avoid NAD^+ depletion under anaerobic conditions. Metabolites other than lactate have been reported as the end-product of hexose fermentation by *L. plantarum*, but only in cases where oxygen or citrate are present as electron acceptors (Ferain *et al.*, 1996b; Lindgren *et al.*, 1990; Tseng & Montville, 1990). Therefore, growth inhibition due to pyruvate accumulation and/or low NAD^+ levels (as a consequence of end-product inhibition by lactate), form a plausible explanation of the inhibitory effect caused by lactic acid. Strategies that aim to avoid pyruvate accumulation and/or regeneration of NAD^+ could be beneficial for *L. plantarum*. The suggested shift towards the phosphoketolase bypass and the pathways leading towards malate and ethanol formation would contribute to a limitation of the pyruvate level. Moreover, these three routes have a clear advantage with respect to cofactor regeneration. These observations indicate that diminished NAD^+ regeneration due to end-product inhibition of the lactate dehydrogenase is an important growth-limiting effect of lactic acid accumulation under anaerobic conditions. This suggestion is confirmed by observations of Ferain and co-workers, who have reported that an *L. plantarum*

Table 7. Genes/operons from group 1 of the hierarchical cluster (Fig. 2)

This group is characterized by the decreased expression of genes/operons under the combination of lactic acid and a higher relative growth rate. Values shown indicate the expression ratios between the condition of interest and the reference condition. Numbers in bold type are within the 95 % confidence interval of the Tukey HSD test. abs. μ , absolute growth rate; rel. μ , relative growth rate.

ORF nos	Assigned function	Mild osmotic stress	Osmotic stress	Acidic pH	Lactate	Lactic acid /higher rel. μ	Lactic acid /lower abs. μ
3611–3614	Sugar transport protein; <i>myo</i> -inositol 2-dehydrogenase; hypothetical protein; oxidoreductase	1.5	0.7	0.5	0.8	0.5	1.3
3604	<i>myo</i> -Inositol transport protein	1.1	0.8	0.4	0.7	0.5	1.3
0746–0750	Phosphate ABC transporter, pstE; pstD; pstC; pstB; pstA	1.2	1.1	0.5	1.1	0.6	1.8
0856	Acyltransferase (putative)	1.1	0.9	0.8	0.7	0.6	1.2
3290	Transcription regulator	0.9	1.0	0.8	0.8	0.5	1.2
1358 or 1359	Integral membrane protein/membrane-bound protease, CAAX family	0.9	0.9	0.7	0.9	0.5	1.1
3283	Hypothetical protein	0.8	1.3	0.9	0.8	0.6	1.2
0325–0327	Transcription regulator; ABC transporter, ATP-binding protein; hypothetical protein	0.7	1.0	0.7	1.0	0.5	1.0
2579	Hypothetical protein	1.0	1.0	1.0	1.0	0.5	0.9
1283	Sulfatase	0.7	0.6	0.9	1.2	0.3	0.9
2732–2733	Oxidoreductase; oxidoreductase	1.4	0.6	0.8	1.0	0.5	1.0
2075–2077	Regulator; nitrate ABC transporter, nrtC; nrtB	1.1	0.6	0.8	1.2	0.6	1.1
1908	Hypothetical protein	0.8	3.0	1.3	1.3	0.2	0.7
0892–0893	Putative regulator; putative transport protein	1.1	1.5	1.0	1.2	0.5	0.9
0343–0344	Teichoic acid ABC transporter, tagG; tagH	1.1	1.1	0.9	1.2	0.4	0.8
3281	Putative permease	0.9	1.5	0.8	0.9	0.4	0.9
2580	Alkaline phosphatase superfamily protein	1.0	1.5	1.0	1.0	0.4	0.7
0871 or 0872	Hypothetical protein/hypothetical protein	0.9	1.3	0.9	1.0	0.4	0.8
0346–0348	Integral membrane protein; putative transcription regulator; permease	1.0	1.3	0.8	1.0	0.5	0.7
2578	Cell surface protein	0.9	1.1	1.1	1.0	0.6	0.8
3468–3469	Lactose transport protein; β -galactosidase	0.8	0.8	2.7	1.0	0.2	0.5
3466	Branched-chain amino acid transport protein	1.0	0.8	1.5	1.0	0.3	0.6

strain that lacks lactate dehydrogenase activity is especially inhibited in its growth under anaerobic conditions (Ferain *et al.*, 1996a). Under aerobic conditions, the NAD⁺-regenerating function of the lactate dehydrogenase could be taken over by NADH oxidase.

The fact that these genes did not show similar trends in response to lactic acid at a higher relative growth rate may indicate that lactic acid is not the only regulatory trigger.

Growth rate-related effects

From clusters 3 and 5 versus clusters 1 and 6 in the hierarchical cluster (Fig. 2), it becomes apparent that altered growth characteristics (in comparison with the reference condition) form an important parameter in gene expression studies.

The coherence within these clusters clearly indicates that the observed changes are not coincidental: the combined effect of lactic acid and a lower absolute growth rate resulted

in, among other effects, the decreased expression of genes involved in transport processes and amino acid uptake, and the previously discussed differential expression of genes involved in metabolic rerouting (Tables 4 and 5). Among the genes that responded to the effect of lactic acid and a higher relative growth rate, increased expression was observed for a remarkably high number of tRNA ligases (Table 6). Based on the fact that the difference in absolute growth rate does not lead to a significant difference in the expression of genes that have been related to glucose limitation (unpublished results), we conclude that this difference does not increase the effect of the glucose starvation on the cells.

Especially in exponentially growing cultures, it is generally difficult to avoid changes in growth rate as a consequence of the imposed stress. The effect of altered absolute growth rate on gene expression is illustrated by three papers on proteome/transcriptome changes in *Escherichia coli* in response to acetate (Arnold *et al.*, 2001; Kirkpatrick *et al.*, 2001; Polen *et al.*, 2003). Transcriptome studies of the effect

Fig. 5. Potential glucose fermentation in *L. plantarum* WCFS1. Block arrows indicate either increased (up arrow), decreased (down arrow) or unchanged (block) expression of the gene in the presence of lactic acid and a lower absolute growth rate. Arrows with a grey filling represent genes for which the change in expression was within the 90 % confidence interval of the Tukey HSD test. (1), phosphoketolase; (2), transaldolase; (3), acetate kinase; (4), transketolase; (5), phosphoenolpyruvate carboxykinase; (6), malate dehydrogenase; (7), pyruvate carboxylase; (8), phosphoenolpyruvate synthase; (9), pyruvate kinase; (10), pyruvate formate-lyase; (11), phosphotransacetylase; (12), bifunctional alcohol dehydrogenase/acetaldehyde dehydrogenase; (13), lactate dehydrogenase.

of a growth-inhibiting concentration of this organic acid on exponentially growing cells reveal a decreased expression for a large number of genes involved in transcription and translation (Arnold *et al.*, 2001). Other studies have recognized this problem and circumvented it by using an acetate concentration that hardly affects growth rate (Kirkpatrick *et al.*, 2001; Polen *et al.*, 2003). However, for most stress-response studies this will not be desirable, since the effects on gene expression will also be limited. Experiments with

steady-state cultures of *E. coli* confirm the effect of absolute growth rate on both metabolome and gene expression (Hua *et al.*, 2004; Tweeddale *et al.*, 1998).

Whereas the use of steady-state cultures with an equal dilution rate appears to be the method of choice to circumvent absolute growth rate-related effects, our data indicate that differences in relative growth rate should also be accounted for.

Table 8. Genes that were differentially expressed under the combination of lactic acid and a lower absolute growth rate and that suggest metabolic rerouting under this condition

See also Fig. 5.

ORF no.	Assigned function	Expression ratio
3538	Transketolase	2.3
3539	Transaldolase	2.3
3551	Phosphoketolase	5.4
1912	Phosphoenolpyruvate synthase	7.0
3418	Phosphoenolpyruvate carboxykinase	3.0
2136	Pyruvate carboxylase	0.6
1897	Pyruvate kinase	0.5
2596	Formate acetyl transferase activating enzyme	3.3
2598	Formate acetyl transferase	1.8
3662	Alcohol dehydrogenase/acetaldehyde dehydrogenase	2.5

Concluding remarks

The experimental design that was applied in this study allowed the different effects of lactic acid to be distinguished, and resulted in new insights into both the effect of lactic acid stress on *L. plantarum* and the potential environmental response mechanisms of the organism. In particular, those genes that show increased expression towards lactic acid regardless of growth rate-related effects may be good target genes for strain improvement. The metabolic reroutings in response to lactic acid at a lower absolute growth rate suggest an inhibitory effect of lactic acid at lower growth rates due to decreased NAD⁺ regeneration and/or pyruvate accumulation. The fact that these effects only occur in combination with the lower absolute growth rate indicates that lactic acid itself is not the regulatory trigger for these reroutings.

The results presented in this paper demonstrate the importance of good experimental design in studies in which more than one physico-chemical parameter is affected by the subject of study. A poor discrimination of primary and secondary effects, such as growth-related effects, will complicate data interpretation and the rational selection of genes for further studies, and should therefore be avoided.

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