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ORIGINAL ARTICLE

Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis

Roberto Mazzoli · Enrica Pessione · Magali Dufour · Valérie Laroute · Maria Gabriella Giuffrida · Carlo Giunta · Muriel Cocaign-Bousquet · Pascal Loubière

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Abstract GABA is a molecule of increasing nutraceutical interest due to its modulatory activity on the central nervous system and smooth muscle relaxation. Potentially probiotic bacteria can produce it by glutamate decarboxylation, but nothing is known about the physiological modifications occurring at the microbial level during GABA production. In the present investigation, a GABA-producing *Lactococcus lactis* strain grown in a medium supplemented with or without glutamate was studied using a combined transcriptome/proteome analysis. A tenfold increase in GABA production in the glutamate medium was observed only during the stationary phase and at low pH. About 30 genes and/or proteins were shown to be differentially expressed in glutamate-stimulated conditions as compared to control conditions, and the modulation exerted by glutamate on entire metabolic pathways was highlighted by the complementary nature of transcriptomics and proteomics. Most glutamate-induced responses consisted in under-expression of metabolic pathways, with the exception of glycolysis where either over- or under-expression of specific genes was observed. The energy-producing arginine deiminase pathway, the ATPase, and also some stress proteins were down-regulated, suggesting that glutamate is not only an alternative means to get energy, but also a protective agent against stress for the strain studied.

Keywords GABA · Glutamate decarboxylase · Branched chain amino acids · ADI route · Stress · ATPase

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R. Mazzoli · E. Pessione (✉) · M. Dufour · C. Giunta
Dipartimento di Biologia Animale e dell'Uomo,
Università di Torino, Via Accademia Albertina 13,
10123 Torino, Italy
e-mail: enrica.pessione@unito.it

R. Mazzoli · V. Laroute · M. Cocaign-Bousquet · P. Loubière
Université de Toulouse; INSA,UPS,INP; LISBP,
135 Avenue de Rangueil, 31077 Toulouse, France

M. G. Giuffrida
ISPA-CNR. c/o Bioindustry Park Canavese, Via Ribes 5,
10010 Colleretto Giacosa, TO, Italy

R. Mazzoli · V. Laroute · M. Cocaign-Bousquet · P. Loubière
INRA, UMR792 Ingénierie des Systèmes Biologiques
et des Procédés, 31400 Toulouse, France

R. Mazzoli · V. Laroute · M. Cocaign-Bousquet · P. Loubière
CNRS, UMR5504, 31400 Toulouse, France

Introduction

Biogenic amines can be found in a great number of different foods, above all those involving a microbial fermentation process (dairy products, wine, beer and fermented sausages). High amounts of these compounds, in particular histamine and tyramine, are toxic for consumers (Millichap and Yee 2003).

Different from the other biogenic amines, γ-aminobutyric acid (GABA) is known to have positive effects on human health; it is the most widely distributed neurotransmitter in the vertebrate central nervous system and it also plays a regulatory and trophic role on the pancreas (Siragusa et al. 2007) besides lowering the blood pressure in mild hypertensive patients (Inoue et al. 2003).

Biogenic amines present in fermented foods, are mainly the products of microorganisms (above all lactic acid

bacteria, LAB) able to decarboxylate precursor amino acids to their respective amines. Biogenic amine biosynthesis by bacteria is usually considered to be involved in two main physiological roles: (1) a defense mechanism to counteract acidic environments (Schelp et al. 2001; van de Guchte et al. 2002) and (2) a means to obtain metabolic energy through electrogenic amino acid/amine antiporters leading to the generation of proton motive force (Higuchi et al. 1997; Konings 2006).

The functional coupling between amino acid decarboxylases and amino acid/amine antiporters is usually reflected by a physical and regulatory association of the genes encoding these proteins (Lucas et al. 2003, 2005). What specifically concerns the glutamate decarboxylative pathway, a structural association between *gadA* and *gadB*, the genes encoding two glutamate decarboxylase isozymes, and *gadC*, the determinant for a glutamate/GABA antiporter, has been described in *E. coli* (Tramonti et al. 2006). The same model can be found, with some variations in the gene number, in Gram-positive organisms: in *L. lactis* there are only two associated genes, *gadB* and *gadC*, encoding a glutamate decarboxylase and an antiporter, respectively (Sanders et al. 1998).

The aim of the present research was to study the metabolic modifications occurring in a *Lactococcus lactis* (the main bacterium involved in cheese production) strain (NCDO2118) during GABA production; a combined transcriptomic-proteomic approach (Cox et al. 2005; Singh and Nagaraj 2006; Vercauteren et al. 2007) was used to compare the physiological responses in growth conditions stimulating GABA accumulation (presence of the precursor amino acid glutamate) versus control conditions.

Materials and methods

Bacterial strain

Lactococcus lactis subsp. *lactis* NCDO 2118 was selected from among the *L. lactis* strains available in the laboratory (LISBP of INSA-Toulouse, France), for its ability to biosynthesize detectable amounts of GABA.

Culture conditions

Bacterial cultures were performed in triplicate in a 2 L fermentor (Setric Génie Industriel, Toulouse, France) in a chemically defined medium (CDM) (Poolman and Konings 1988), glutamate-free, and in the same medium supplemented with 5 g L^{-1} (34 mM) glutamic acid. Cultures were incubated at 30°C in anaerobiosis, obtained by slight N_2 overpressure. The pH was maintained at 6.6 by addition of KOH until the cultures reached $OD_{580} = 1$, in order to reach enough biomass for further analytical procedures, and then pH was not regulated anymore.

Bacterial growth was estimated by spectrophotometric measurements at 580 nm (Hitachi U1100, 1 absorbance unit = 0.3 g L^{-1}). For transcriptomic and proteomic analyses, the biomass was harvested 30 h after culture inoculation in the late stationary phase consistent with maximum GABA production.

Metabolite determination

Concentrations of glucose and metabolic products (lactate, acetate, formate and ethanol) in the culture supernatants were measured by HPLC as previously described (Novak et al. 1997).

Free amino acids and the GABA concentration in the culture supernatants were measured via the AminoQuant HP1090 system (HP 1090; Hewlett Packard, Waldbronn, Germany). Proteins in the samples were precipitated by adding four volumes of methanol followed by overnight incubation on ice. The mixture was centrifuged and the supernatant kept for amino acid analysis. The amino acids were automatically derived with OrthoPhtalic Aldehyde (OPA) and 9-fluorenylmethyl-chloroformiate (FMOC-C1). The derivatives were separated on a Hypersil AA-ODS column (Agilent Technologies) at 40°C by a linear gradient of acetate buffer (pH 7.2) with triethylamine (0.018%), tetrahydrofurane (0.3%) and acetonitrile (60%). A diode array detector was used at 338 nm for OPA derivatives and 262 nm for FMOC derivatives.

Proteomic analysis

Cell lysis

Equivalent amounts of cells (25 mg dry weight for each experiment) were treated in each protein preparation. Cells were collected by centrifugation (5,000*g*, 15 min, 4°C) and washed twice in 50 ml 0.85% NaCl. The biomass was re-suspended in 3 mL 50 mM Tris–HCl pH 7.3, 1 mM EDTA and sonicated as previously described (Pessione et al. 2005). Unbroken cells were sedimented by centrifugation (3,000*g*, 20 min, 4°C), re-suspended in the same buffer, sonicated and centrifuged again as described above. The supernatants were pooled and ultracentrifuged (100,000*g*, 8 h, 4°C) to precipitate cell envelope components. The supernatant (soluble fraction) and the pellet (membrane fraction) were harvested and treated separately as described in the following paragraphs.

Preparation of soluble extracts

Soluble extracts were supplemented with ribonuclease A and deoxyribonuclease I mixture (94 units; GE Healthcare) and incubated at room temperature for 20 min. The samples were dialyzed against three volumes of water for three times. Proteins were precipitated with methanol/chloroform (1:2) to remove salts and lipids and re-suspended in 7.0 M urea, 2.0 M thiourea, 4% CHAPS, 20 mM Tris, 0.5% IPG buffer and 100 mM DTT (Rabilloud 1998). Protein concentration was evaluated by 2D Quant kit (GE Healthcare).

Extraction of membrane proteins

The membrane proteins were extracted according to Zuobi-Hasona et al. (2005), with some modifications as described by Pessione et al. (2009).

2-DE

The protocol of Pessione et al. (2005) was used with some modifications. Briefly, IEF was performed using 13-cm pH 4–7 L or 18-cm pH 4.2–5.6 NL IPG strips (GE Healthcare). Protein extracts (300–400 µg) were loaded by the in-gel re-hydration method. IEF was performed using IPGphor (GE Healthcare) at 20°C with 66,000 Vhs after 12 h of re-hydration. Before the second dimension, proteins were reduced and alkylated (Giuffrida et al. 2001). SDS-PAGE was carried out on 11.5% polyacrylamide Duracryl (Genomic Solutions Chelmsford, MA, USA) or on 9.5% polyacrylamide Bio-Rad (Hercules, CA) homogeneous running gels, so as to obtain optimal resolution of low and high M_r proteins, respectively. The running buffer and conditions were the same as described by Pessione et al. (2005). Gels were automatically stained using Processor Plus (GE Healthcare) with freshly prepared colloidal Coomassie blue (Neuhoff et al. 1988). The 2-DE gels were digitized and image analysis was performed with Image Master 2D Elite v3.1 software (GE Healthcare), as previously described (Pessione et al. 2005).

Statistical analysis

Two biological replicates were analyzed and three technical replicates were performed for each 2-DE gel. Spot intensities were measured via spot volumes with or without all spot mean normalization. Spot intensities were statistically analyzed by the *t* test: means were considered significantly different when $P < 0.05$.

Protein identification

Spots of interest were cut from the gel and destained overnight with a solution of 25 mM ammonium bicarbonate and 50% acetonitrile. The proteins were digested "in gel" with trypsin (Promega, Madison, WI, USA) as previously described (Pessione et al. 2005).

The tryptic peptide mixtures were analyzed by LC–MS/MS using an Agilent (Palo Alto, CA, USA) 1100 series nano HPLC coupled to an Agilent XCT Plus ion trap fitted with a nano electrospray nebulizer. The chromatographic separations were run on a C18 nanocolumn Zorbax 300SB 150 × 0.075 mm (Agilent, Palo Alto, CA, USA). Injection volume was 1 µL and flow rate 300 nL min^{-1}. Gradient mobile phase composition was adopted: 95/5 for the first 10 min to 45/55 in 35 min and then to 70/30 in 5 min. Eluent A was distilled water with 0.1% formic acid and 5% acetonitrile, and eluent B was acetonitrile with 0.1% formic acid. Capillary voltage was 1,600 V and fragmentation voltage was 1.3 V.

For the elaboration of the LC and MS/MS data, the Data Analysis software (Agilent, Palo Alto, CA, USA) was used. The Mascot search engine (Matrix Science Ltd, London UK, free online) was used for identifying the proteins against the NCBInr_20090123 database. The parameters used for the search were as follows: S-carbamido methyl cysteine as fixed modification, oxidized methionine as variable modification and two missed cleavage sites for trypsin digestion. Peptide mass tolerance was set up to 0.5 Da and fragment mass tolerance to 0.8 Da. The criteria used for protein identification were: 20 ion score, protein Mascot score more then 100 and at least two unique peptides sequenced.

Transcriptomic analysis

RNA extraction

Cells (6 mg of dry weight) were harvested and frozen immediately in liquid nitrogen. RNA was extracted as previously described (Redon et al. 2005) and quantified at 260 and 280 nm. RNA quality was controlled on denaturing agarose gel electrophoresis and on Agilent Bioanalyzer.

cDNA preparation

The cDNA preparation and purification were performed using LabelStar Array kit (Qiagen) as previously described (Maligoy et al. 2008). The cDNAs of cells grown with and without glutamate were labeled with cyanine3-dCTP and cyanine5-dCTP and inversely. The dye-switch, avoiding differential efficiency of incorporation of the two labeled

nucleotides, was performed with two methodological replicates. Two independent biological repetitions were analyzed.

Hybridization of labeled cDNA and scanning the glass slides

Glass slides contained the *L. lactis* IL1403 DNA probes set provided by Eurogentec (2005 of the 2310 ORFs identified in the genome). Experiments were carried out at the Biochip platform of Toulouse Genopole with an automatic hybridization chamber (Discovery, Ventana Medical System, Inc.). Pre-hybridization occurred at 42°C and hybridization was performed as previously described (Maligoy et al. 2008) with 10 µL of Cy3-labeled cDNA and 10 µL of Cy5-labeled cDNA corresponding to the two culture conditions. Fluorescence signals (at 532 nm for Cy3-labeled cDNA and 635 nm for Cy5-labeled cDNA) were captured with a laser scanner (GenePix 4000A; Axon Instrument, CA, USA). Spot detection was performed with GenePix 6.0 software.

Statistical analysis

Expression ratios were calculated using the control without glutamate as reference. Four repetitions were available: two biological repetitions and two methodological repetitions corresponding to the dye-switch. Hybridization signals were statistically treated with the Bioplot software (developed by S. Sokol in the Biochip Platform, Toulouse, see http://biopuce.insa-toulouse.fr). Signals were normalized by the Lowess method and differentially expressed genes were identified by the Student's *t* test. False discovery rate (FDR) was calculated by the Storey method and the list of genes with an FDR lower than 10% was selected.

Results

Effect of glutamate on growth and metabolism

Fermentation profiles and GABA accumulation by *L. lactis* NCDO 2118 were investigated in CDM (10 g L^{-1} glucose) in the presence and the absence of glutamate, i.e., the precursor amino acid for GABA production.

In both glutamate-fortified and glutamate-free conditions, growth started immediately after inoculation with maximal growth rates of 1.07 ± 0.05 and 0.97 ± 0.09 h^{-1}, respectively. Growth stopped, in both conditions, after 4.5 h at 0.75 g L^{-1} biomass, which was maintained during the whole stationary phase (Fig. 1). Irrespective of glutamate, about half of the glucose was consumed during the growth phase, and it continued to be consumed during the stationary phase, although it was not exhausted after 30 h of culture. The metabolism remained homolactic all along the culture (data not shown).

In both conditions, the pH decreased to reach a value of around 4.4 at the growth arrest. Afterwards the presence of glutamate seemed to slightly reduce medium acidification since the final pH (measured after 24 h of culture) was 4.16 ± 0.01 and 4.00 ± 0.03, with and without glutamate, respectively.

The provided amino acids were consumed, though at different amounts, exclusively during the growth phase (data not shown). That was particularly the case for glutamine (initial concentration = 3.6 mM): 0.6 mM was

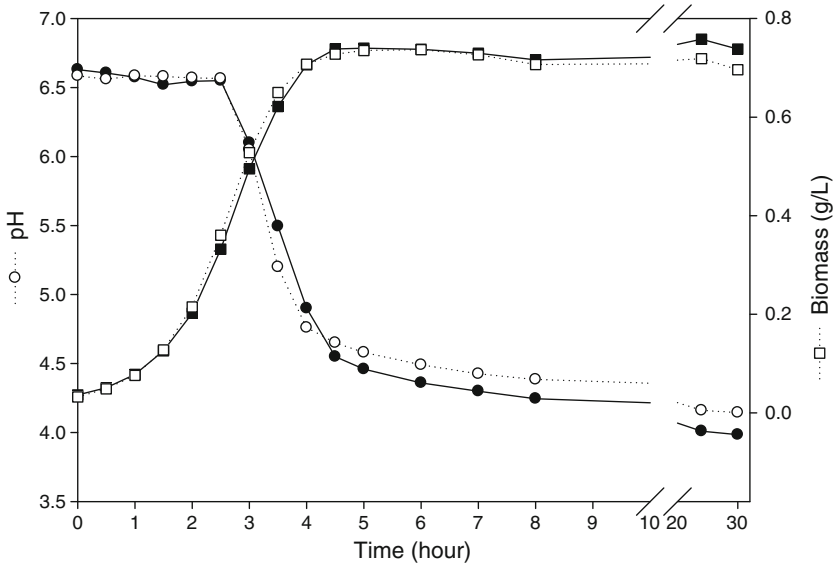

Fig. 1 Evolution of biomass (*square*) and pH (*circle*) during growth of *L. lactis* NCDO 2118 on CDM with 5 g L^{-1} of glutamate (*dotted lines*) or without glutamate (*solid lines*)

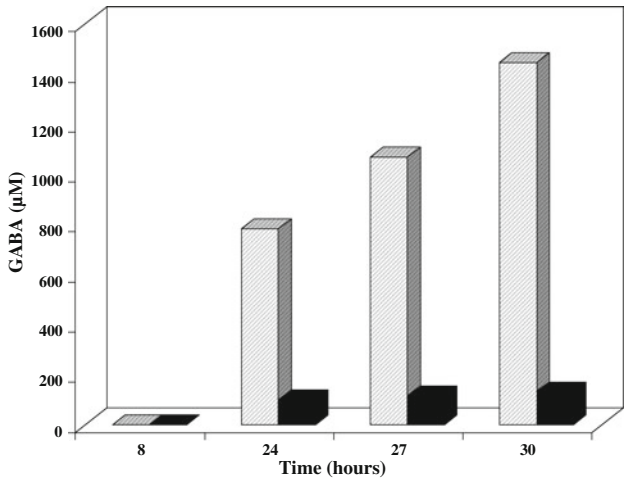

Fig. 2 GABA concentrations detected at different cultivation time during growth of *L. lactis* NCDO 2118 on CDM with 5 g L^{-1} of glutamate (*gray*) or without glutamate (*black*)

consumed during the logarithmic growth phase while no significant consumption was observed during the stationary phase. Without added glutamate, a low concentration of GABA, i.e., about 0.13 mM, was detected in the late stationary phase, probably resulting from glutamine conversion into glutamate and its subsequent decarboxylation into GABA. In the presence of glutamate, besides glutamine consumption, 2.3 mM of glutamate was consumed throughout the culture. This glutamate consumption led to the production of 1.5 mM of GABA only during the late stationary phase (Fig. 2). Moreover, no GABA production was detected, even in the stationary phase, in the cultures where the pH was regulated at 6.6 (data not shown), suggesting that both stationary phase and a low pH are necessary for GABA biosynthesis.

Transcriptome analysis

The transcriptome of both glutamate-containing and glutamate-free cultures was analyzed 30 h after inoculum. Only 27 genes were differentially expressed, 5 being over- and 22 under-expressed in the presence of glutamate (Table 1). The mRNA ratios between stimulated and control conditions ranged between 0.63 and 1.44 only. Three over-expressed genes encoded phage related or unknown functions, and two were glycolytic genes (*yjhF* and *gapA*); *gapA* encodes an auxiliary glyceraldehyde-3-phosphate dehydrogenase (GAPDH) active during particular growth conditions (Willemoës et al. 2002). On the contrary, four glycolytic genes including *gapB*-encoded GAPDH and ldh were under-expressed in the presence of glutamate. In the same condition, three genes encoding enzymes involved in the arginine deiminase (ADI) pathway were also under-expressed, together with four ATPase subunits and two stress proteins encoding genes (Table 1).

Proteome analysis

The same samples used for transcriptomic analyses were submitted to proteome evaluation. Since the pI of most proteins of *L. lactis*, as predicted by its genome sequence, lies in the acidic pH range, we firstly performed pH 4–7 maps (Fig. 3a, b). Image analysis revealed three down-expressed proteins (ornithine carbamoyltransferase, adenylosuccinate synthetase and 3-oxoacylcarrier protein reductase) and five over-expressed proteins in the presence of glutamate (GAPDH B, non-heme iron binding ferritin, pyrimidine operon regulatory protein, a regulatory protein and an unknown protein) (Table 1). Unfortunately some proteins could not be identified because of their very low amount and there were also many areas with overlapping spots. To improve the resolution of these areas, zooms in the 4.2–5.6 (Fig. 3c, d) and 5–6 (Fig. 3e, f) pH range were performed. One protein previously detected among differentially expressed spots was confirmed and also new spots, corresponding to proteins involved in replication, transcription, translation, nucleic acid metabolism and glycolysis, were identified (Table 1). Although GABA is mainly produced by glutamate-fortified cultures, glutamate decarboxylase (GAD) was not among the soluble proteins induced in this condition.

To verify if GAD could be associated with cell membrane, membrane extracts were analyzed by 2-DE as well (Fig. 4). Even in this fraction, no evidence of a differential GAD expression was shown. However, a modulation of eight proteins, all under-expressed in the presence of glutamate, including a glutamyl amino peptidase (PepA), two glycolytic enzymes (Pyk and Pgk), two enzymes of the central metabolism (L-LDH and PflA) and one of the ADI pathway, was observed (Table 1). Among these eight membrane proteins, three were also identified in the soluble fraction.

Finally, taking into account all the soluble and membrane gels analyzed, a total of 30 proteins appeared to be differentially expressed between the two culture conditions studied.

Discussion

Biogenic amine biosynthesis by LAB usually occurs in response to adverse conditions, as a means to counteract acidity (van de Guchte et al. 2002) and to obtain metabolic energy (Konings 2006). As far as we know, most literature studies concerned toxic amines for human health, such as tyramine and histamine. The present research demonstrated

Table 1 Ratio of mRNAs and proteins at 30 h of growth of *Lactococcus lactis* NCDO 2118 on CDM medium containing 5 g L^{-1} glutamate as compared to the same medium without glutamate. Proteome analysis was performed on different pI range for cytosolic and membrane proteins

	GI #	Transcriptomic analysis mRNA Ratio	Proteomic analyses							
			pI 4–7 cytosol		pI 4.2–5.6 cytosol		pI 5–6 cytosol		pI 4–7 membrane	
			Spot n	Ratio	Spot n	Ratio	Spot n	Ratio	Spot n	Ratio
ADI pathway										
ornithine carbamoyltransferase (*arcB*)	12725085	0.63	172	0.63					405	0.35
Arg/Orn antiporter ($arcD_1$)	12725084	0.80								
Carbamate kinase ($arcC_2$)	12725082	0.63								
Arginine deiminase (*arcA*)	8250402				77	0.28				
ATPases										
ATP synthase b (*atpF*)	12724791	0.81								
ATP synthase δ (*atpH*)	12724790	0.75								
ATP synthase γ (*atpG*)	12724787	0.64								
ATP synthase α (*atpD*)	12724786	0.7								
Glycolysis										
Pyruvate kinase (*pyk*)	12724313	0.71			42,45	0.32			104,109,407	0.27
Phosphoglycerate kinase (*pgk*)	12723104	0.81			127	0.18			454	0.26
Fructose-bisphophate aldolase (*fbaA*)	12724945	0.69			163,164,166	0.52				
Phosphoglycerate mutase (*yjhF*)	12723886	1.23								
GAPDH (*gapA*)	12723446	1.44								
GAPDH (*gapB*)	12725315	0.82	180	1.45	210	4.00				
6-Phosphofructokinase (*pfk*)	15673315						85	1.26		
Phosphoglyceromutase (*pmg*)	15672318						103	1.31		
Aerobic metabolism										
Pyruvate oxidase (*poxL*)	12725094	0.73								
Oxidoreductase (*ycgG*)	15672250						73	1.4		
Anaerobic/fermentation										
L-LDH (*ldh*)	12724312	0.76							293	0.22
Pyruvate formiate lyase (*pflA*)	116512687								575	0.02
Oxidoreductase (*ylbE*)	12724068	0.79								
Amino acid and peptide metabolism										
BCAA aminotransferase (*ilvE*)	125624011						72	1.43		
Glutamyl aminopeptidase (*pepA*)	15672369								176,555	0.34
Stress proteins										
CtsR (*Ctsr*)	12723545	0.80								
ATP-dependent Clp protease proteolytic subunit (*clpP*)	12723580	0.58								
Superoxide dismutase (*sodA*)	809037				193	0.84				
Carrier proteins										
Mannose PTS system IIAB (*ptnAB*)	15673688						64	4.34		
Non-heme iron-binding ferritin (*dpsA*)	15674007		356	2.74						
Pyrimidine metabolism										
Pyrimidine operon regulator (*pyrR*)	12724611		310	1.19						
Orotate phosphoribosyltransferase (*pyrE*)	15673050								497	0.39
CTP synthase (*pyrG*)	15672473						5	4		
Purine metabolism										
Hypoxanthine–guanine phosphorybosyltransferase (*hpt*)	15672002				196	0.19				
GMP synthase (*guaA*)	15673468				37	0.40				
Adenylosuccinate synthetase (*purA*)	15673934		135	0.75						
Lipid metabolism										
3-Oxoacyl-acyl carrier protein synthase II (*fabF*)	15672757						33	1.24		

Table 1 continued

	GI #	Transcriptomic analysis mRNA Ratio	Proteomic analyses							
			pI 4–7 cytosol		pI 4.2–5.6 cytosol		pI 5–6 cytosol		pI 4–7 membrane	
			Spot n	Ratio	Spot n	Ratio	Spot n	Ratio	Spot n	Ratio
3-Oxoacyl-acyl carrier protein reductase (*fabG1*)	15672756		282	0.72						
Ribosome										
50S Ribosomal protein L10 (*rplJ*)	15673251								367	0.37
30S Ribosomal protein S2 (*rpsB*)	116513100						84	1.53		
30S Ribosomal protein S5 (*rpsE*)	12725134	0.83								
Translation - post-translation proteins										
Seryl tRNA synthase (*serS*)	15673694						59	0.7		
Elongation factor EF-Tu (*tuf*)	12724893	0.72								
Replication transcription										
DNA polymeraseIII. β chain (*dnaN*)	15671984				96	0.37				
Phage related functions and prophages										
Prophage pi3 protein 18 (*pi318*)	12724378	1.21								
Transposase (*yajE*)	12722932	0.75								
Regulatory proteins										
Regulatory protein (*ccpA*)	13591401		197a	1.63						
Unknown										
yaiB	12722921	1.28								
yaiA	12722920	1.26								
yldE	12724086	0.83								
yrjF	12724757	0.73								
ywcC	12725214	0.65								
Unknown (*ytcC*)	15673838		197b	1.24						

Only significantly differently expressed genes are reported: for mRNAs this corresponds to genes with $P < 0.05$ (*t* test) and FDR < 10%; protein spot intensities were considered significantly different when $P < 0.05$ (*t* test)

Spot n protein spot number on the corresponding 2-DE gel

that also the decarboxylation of glutamate into GABA by *L. lactis* NCDO 2118 never occurs at neutral or moderately acidic (higher than 5.7) pH, irrespective of the presence of a high glutamate concentration, or during the growth phase, even when the pH decreased until 4.4.

The logarithmic growth phases of *L. lactis* NCDO 2118 on glutamate-fortified and glutamate-free CDM showed nearly identical growth and pH profiles and no GABA production, indicating that glutamate did not significantly affect cell metabolism in this phase. Growth arrest was probably due to inhibition by the acidic pH (4.4) reached in both conditions, since in pH-regulated conditions the CDM is able to support growth until glucose depletion and a biomass concentration twofold higher than what was observed in this study (Novak et al. 1997). Only during the stationary phase in the presence of glutamate the pH decrease was slightly attenuated. It is tempting to hypothesize that this phenomenon is related to GABA production, also considering that LAB generally lack the NH_3 producing glutamate dehydrogenase. However, only 1.5 mM of GABA was produced until 30 h of culture, while at the same time about 20 mM of lactate was synthesized (data not shown). Further investigations are therefore necessary to identify the true mechanism involved in this lower pH decline.

GABA production was enhanced about 10-fold in the presence of glutamate in the medium but, even in these conditions, only about 5% of the glutamate was converted into GABA after 30 h of culture. Clearly, besides glutamate concentration, other parameters affect the efficiency of the glutamate decarboxylative reaction in vivo, including the GAD concentration and its catalytic characteristics (optimal pH, K_M for its substrate, etc.).

To determine whether the expression of *gadB*, encoding GAD, was induced by glutamate, expression patterns of *L. lactis* NCDO 2118 grown in glutamate-free and glutamate-fortified conditions were compared at both transcript and protein levels. Concerning proteome analyses, both soluble and membrane extracts were considered, since recent results proved the presence of another amino acid decarboxylase, i.e., tyrosine decarboxylase, in the

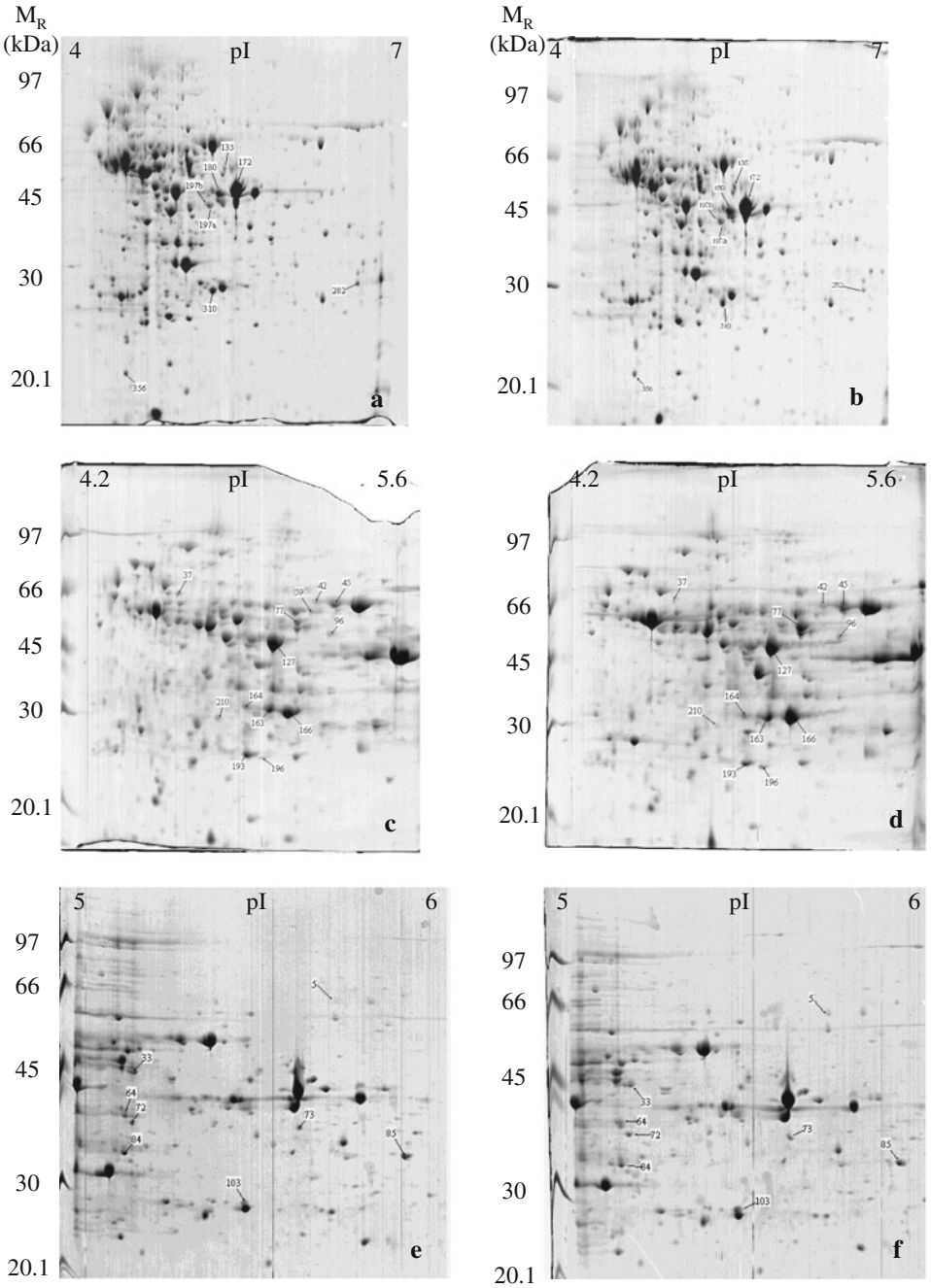

Fig. 3 2-DE maps of cytosolic extracts of *L. lactis* NCDO 2118 grown on CDM with 5 g L^{-1} of glutamate (**b**, **d**, **f**) or without glutamate (**a**, **c**, **e**): pI 4–7 (**a**, **b**), pI 4.2–5.6 (**c**, **d**), pI 5–6 (**e**, **f**). Numbers indicate spots identified by MS

membrane proteome of a tyramine producing *Enterococcus faecalis* strain (Pessione et al. 2009). Proteomic analyses on both sub-proteomes failed to detect GAD among the differentially expressed proteins. These results do not definitely exclude that GAD expression was modulated by glutamate: GAD could be among the unidentifiable spots (spots in too low amounts) or rather, post-translation modifications could have drifted GAD pI in a pH range not considered by the present investigation. Nonetheless transcriptomic analyses showed comparable *gadB* mRNA levels in glutamate-free and glutamate-fortified conditions. The overall findings, together with the observation that low but detectable levels of GABA accumulated even in control conditions, suggest that, at least in this strain, catalytic activation of GAD by glutamate is more important than biosynthetic regulation. This result is in contrast to what was observed for other amino acid decarboxylases in LAB (Pessione et al. 2005; Fernández and Zúñiga 2006; Pessione et al. 2009).

In general, a light tuning of gene expression was observed between glutamate stimulated and control cultures, since for only 27 mRNAs and 30 proteins, significant

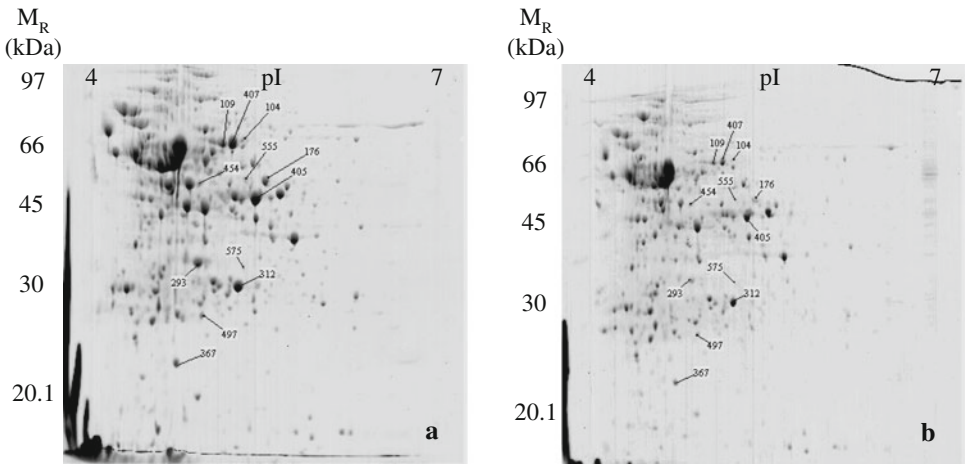

Fig. 4 2-DE maps (pI 4–7) of membrane extracts of *L. lactis* NCDO 2118 grown on CDM with 5 g L^{-1} of glutamate (**b**) or without glutamate (**a**). Numbers indicate spots identified by MS

modulations of the expression were detected. A differential expression was identified via both transcriptome and proteome analyses for six genes only for which results were generally consistent except for GAPDH B that was under-regulated at the transcriptomic level but up-regulated at the protein level. These findings suggest that a significant modulation of translation efficiency occurred for most of the genes considered. However, the complementation of transcriptome and proteome results allowed reconstructing entire metabolic pathways, thus elucidating the regulation exerted by glutamate on them.

Among up-regulated proteins, the identification of the branched-chain amino acid aminotransferase (*ilvE*) reveals glutamate stimulation over leucine, isoleucine and valine metabolic routes. Glutamate is indeed generally involved in transamination as a highly efficient amino group donor for branched-chain amino acid biosynthesis. It is also worth noting that in LAB branched–chain amino acids are among the most abundant amino acids in the cell (Novak et al. 1997).

For what concerns glycolytic enzymes, the experimental results did not reveal a true down- or up-regulation on the overall pathway, but rather a specific modulation for each of them (Table 1). In particular, the down-regulation of pyruvate kinase can cause an accumulation of phosphoenolpyruvate (PEP). It has been suggested (Thompson 1987) that an allosteric attenuation of pyruvate kinase, resulting in a slow utilization of PEP, can contribute to maintain highly efficient PTS systems (PEP-mediated phosphotransferase) in LAB. In the present investigation, we observed a biosynthetic activation of a mannose-specific PTS system component (*ptnAB*, detected with proteomics) in presence of glutamate.

The finding of two glycolytic enzymes in the membrane proteome could account for a certain degree of cytosolic protein contamination, even if for one of them (pyruvate kinase) previous reports stress frequent membrane localization (Raikar et al. 2006). For what specifically concerns bacteria, fructose-bisphosphate aldolase and phosphoglucomutase as well as phosphoglycerate kinase have been reported to be surface-located in *Streptococcus oralis* (Wilkins et al. 2003) and group B streptococci (Hughes et al. 2002) suggesting that a displacement of glycolytic enzymes is not unusual in LAB.

Both proteomic and transcriptomic results indicated that glutamate triggers a down-regulation of proteolytic enzymes such as those encoded by *clpP* and *pepA*. It is known that some proteases are stress-related: the cellular stress responses include the rapid and transient induction of proteolytic activities to cope with the accumulation of damaged proteins. In *L. lactis*, *clpP* encodes the proteolytic subunit of Clp-protease that is central in both the proteolysis of misfolded proteins and in adjusting the level of key regulatory proteins in the cell (Frees et al. 2007). The observed down-regulation of ClpP therefore suggests that glutamate can attenuate stress, and in particular stationary phase stress. A confirmation of this hypothesis comes from the finding that also SOD, a stress marker, is down-expressed in glutamate-stimulated cultures (Table 1). The action of glutamate as an osmoprotectant has been described in bacteria: high glutamate concentrations, like those supplied and not metabolized by *L. lactis* in the present study, are required to maintain internal K^+ level (Yan 2007) and to protect LAB from freezing and freeze-drying stress (Martos et al. 2007). On the contrary, the glutamate induced down-expression of the protease PepA found in the membrane fraction, seems rather linked to its peculiar peptidase activity and its very high substrate specificity: it releases N-terminal acidic residues (glutamate) from peptides (Exterkate and de Veer 1987; Kim et al. 2009), so its presence probably becomes completely useless when glutamate is already present in high concentration in the culture medium.

It is also worth noting the under-expression, in glutamate-stimulated conditions, of both ATP synthase (*atpF*,

atpG, *atpH* and *atpD*) and ADI pathway (*arcA*, *arcD1*, *arcB* and *arcC2*) genes. The ADI pathway down-regulation by glutamate suggests that glutamate decarboxylation and arginine deimination are competing routes. Both produce energy, in the form of PMF or ATP, respectively, and both counteract acidity by means of alkaline metabolites extrusion (GABA or ammonia, respectively). A number of systems allowing maintaining the intracellular pH have previously been characterized in LAB, namely, the F_0F_1-ATPase (Konings 2002), the glutamate decarboxylase—GABA antiporter (Sanders et al. 1998) and, in some *L. lactis* strains, the ADI pathway (Budin-Verneuil et al. 2004). The low expression of the β subunit of F_0 (*atpF*) suggests that the cells are decreasing proton pumping because protons are already extruded via the glutamate/GABA antiporter. In parallel, the down-regulation of the F_1 component (*atpG*, *atpH* and *atpD*) indicates that the potential energy, supplied as PMF by the glutamate/GABA antiporter, is not necessarily driven to ATP biosynthesis but rather is used to help nutrient uptake or to accomplish other cellular works.

Further evidence coming from the present investigation is the modulation of pyrimidines anabolism observed in stimulated conditions: *pyrE* was negatively controlled in glutamate-fortified conditions while *pyrG* and *pyrR* were over-expressed. This last acts as an attenuator of transcription of the pyrimidine biosynthetic operon, in which *pyrE* (orotate phosphoribosyl transferase) is a structural gene. *PyrG* (CTP synthase) is a separately regulated gene not belonging to the *pyr* operon (Jorgensen et al. 2004). Thus, it seems that glutamate, or some metabolic events connected with its bio-transformations, exert inhibitory control over the first steps of pyrimidine biosynthesis, but not over the conversion UTP/CTP, where activation is observed on CTP synthase. On one hand, this finding is in agreement with the above comments: carbamoyl-phosphate, the first substrate for pyrimidine biosynthesis, is also an end-product of the ADI route. Considering the ADI pathway inhibition in the presence of glutamate, it is quite probable to suppose a lack of its end-product. The reduced carbamoyl-phosphate pool can thus in someway limit pyrimidine anabolism, as previously demonstrated by Nicoloff et al., in *Lactobacillus plantarum* (Nicoloff et al. 2005). On the other hand, recently (Arsène-Ploetze et al. 2006) there has been evidence that *pyr* operon is transcriptionally modulated by inorganic carbon (carbon dioxide, bicarbonate), so not only carbon dioxide deriving from carbamoyl-phosphate, but also the one released by glutamate decarboxylation could play a role in modulating this biosynthetic route.

In conclusion the present investigation has shed light on some metabolic modulations occurring in *L. lactis* NCDO 2118 during GABA production, revealing that no significant stimulation of pathways generating toxic metabolites takes place. On the contrary it seems that the ammonia generating ADI route is lightly negatively regulated, thus opening the possibility to use this strain for probiotic applications.

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