Amino Acids (2011) 41:517–527
DOI 10.1007/s00726-010-0781-2

ORIGINAL ARTICLE

ADI pathway and histidine decarboxylation are reciprocally regulated in *Lactobacillus hilgardii* ISE 5211: proteomic evidence

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Received: 9 June 2010 / Accepted: 1 October 2010 / Published online: 26 October 2010

Abstract Amine production by amino acid decarboxylation is a common feature that is used by lactic acid bacteria (LAB) to complement lactic fermentation, since it is coupled with a proton-extruding antiport system which leads to both metabolic energy production and the attenuation of intracellular acidity. Analogous roles are played in LAB by both malolactic fermentation (MLF) and the arginine deiminase (ADI) pathway. The present investigation was aimed at establishing reciprocal interactions between amino acid decarboxylation and the two above mentioned routes. The analyses were carried out on a *Lactobacillus hilgardii* strain (ISE 5211) that is able to decarboxylate histidine to histamine, which had previously been isolated from wine and whose complete genome is still unknown. The 2DE proteomic approach, followed by MALDI TOF–TOF and *De Novo* Sequencing, was used to study the protein expression levels. The experimental evidence has indicated that malate does not influence histidine decarboxylase (HDC) biosynthesis and that histidine does not affect the malolactic enzyme level. However, the expression of the ADI route enzymes, arginine deiminase and ornithine transcarbamylase, is down-regulated by histidine: this biosynthetic repression is more important (4-fold) in cultures that are not supplemented with arginine, but is also significant (2-fold) in an arginine supplemented medium that normally induces the ADI pathway. On the other hand, arginine partially represses HDC expression, but only when histidine and arginine are both present in the culture medium. This proteomic study has also pointed out a down-regulation exerted by histidine over sugar metabolism enzymes and a GroEL stress protein. These data, together with the reciprocal antagonism between arginine deimination and histidine decarboxylation, offer clue keys to the understanding of the accumulation of lactate, amine, ammonia and ethylcarbamate in wine, with consequent implications on different health risk controls.

Keywords LAB · HDC · MLF · ADI pathway · 2DE

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Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0781-2) contains supplementary material, which is available to authorized users.

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Introduction

Lactic acid bacteria (LAB) are Gram-positive microorganisms that are involved in several industrial processes, such as fermented food production (Topisirovic et al. 2006) and lactic acid production for biodegradable polymer synthesis (Lim et al. 2008; Bhuvanesh et al. 2007). They chiefly gain metabolic energy by converting lactose into lactate (lactic fermentation) through the glycolytic pathway (homofermentative species) or by means of the phosphoketolase reaction through the pentose-phosphate route

(heterofermentative species). Their ability to produce ATP through respiration is strongly limited since most of them cannot biosynthesize heme, the essential component of cytochromes; they can activate therefore only functional respiratory chains with external hemine supplementation (Vido et al. 2004). For these reasons, LAB have developed systems, during evolution, to obtain supplementary energy, by-passing the respiratory activity and complementing homo- or hetero-lactic fermentation.

Among these systems the most fully characterized are: (1) the decarboxylation of organic acids (chiefly the one that converts malate into lactate; Poolman et al. 1991); (2) pyridoxal phosphate (PLP)—or piruvoyl-dependent decarboxylation of amino-acids that gives rise to amines (Konings et al. 1995). Both decarboxylative activities are coupled with electrogenic antiport systems (catalyzing dicarboxylic–monocarboxylic acid or amino acid/amine exchange across the cell membrane) that generate a protonmotive force (PMF) (Molenaar et al. 1993). Genetic determinants for decarboxylative enzymes and antiporters are often encoded by the same operon (Lucas et al. 2003). The decarboxylation of some dicarboxylic acid to monocarboxylic acids (i.e. malate conversion into lactate, catalyzed by the malolactic enzyme), is appreciated in the food industry, as it results in the attenuation of acidity, especially in red wines (malolactic fermentation or MLF) (Kunkee 1967). On the contrary, amino acid decarboxylation should be avoided since the accumulation of amines can alter the organoleptic properties of food and in some cases may cause severe syndromes (Millichap and Yee 2003). Theoretically, all fermented foods (wine, beer, cheese, fermented sausages, sauerkrauts) are subject to the risk of amine contamination: this risk can be either higher or lower, depending on the food with free amino acid content. This contamination is the result of the proteolytic activity of the starter strain(s) or other contaminant organisms that are able to live in the same ecological niche (Alexandre et al. 2001). For instance, wine usually contains a high arginine concentration but other amino acids (i.e. histidine, tryptophane, lysine, glutamate) can also be present and released during yeast autolysis occurring after alcoholic fermentation (Alexandre et al. 2001). Cheese can exhibit high concentrations of tyrosine or phenylalanine, depending on both the microbial ecosystem established during manufacturing and the proteolytic activity which is necessary for the maturation steps (Joosten 1988a, b).

Together with these energy-supplying decarboxylative reactions, LAB can also obtain ATP from the arginine deiminase pathway (ADI pathway), a metabolic route which generates ammonia (useful in pH control during growth), carbamoyl phosphate (which can be either used as a precursor for pyrimidine biosynthesis or hydrolyzed to obtain ATP) and ornithine. The latter can be further decarboxylated into putrescine leading to the production of PMF through an electrogenic ornithine/putrescine antiporter (Arena and Manca de Nadra 2001)

Since the strategies described above constitute a redundant means of obtaining energy, it was considered interesting to investigate whether bacterial cells use alternative metabolic routes competing against each other or simultaneously activate all of them as a global response to the higher energy supply requirement.

Although some previous papers have related carbon catabolite (glucose, lactate or malate) repression to amino acid decarboxylative enzymes (Lonvaud-Funel 2001; Landete et al. 2006) and/or to the ADI pathway (De Angelis et al. 2002; Mangani et al. 2005), to the best of the authors' knowledge the effect of free amino acids on the ADI pathway and MLF has been poorly elucidated.

In previous papers, the authors demonstrated that amine accumulation in LAB, through histidine and ornithine decarboxylative activity, depends on the presence of high concentrations of the two precursor amino acids in the culture medium (histamine and ornithine, respectively) (Pessione et al. 2005), and that arginine can delay histamine production in *L. hilgardii* ISE 5211 (Mazzoli et al. 2008).

The aim of the present investigation was to evaluate the reciprocal relationships between histidine decarboxylation, MLF and the ADI pathway, in *L. hilgardii* ISE 5211 (isolated from an Italian red wine that had undergone spontaneous MLF) grown in media supplemented with histidine and/or arginine and/or malate. A comparative proteomics strategy was employed as it is useful to detect biosynthetic modulations of the enzymes involved in the relative pathways.

Since histamine accumulation in wines can cause serious diseases such as respiratory distress, hypotension, headaches and allergic disorders (Silla Santos 1996), and metabolites resulting from the ADI pathway are responsible for even more severe consequences (ammonia is toxic; ethyl carbamate, produced by the reaction of carbamoyl phosphate with ethanol, is carcinogenic; Terrade and Mira de Orduna 2006), the final objective of this study was to elucidate the environmental features that could interfere with these metabolic pathways, and, as a consequence, to contain the health risks connected to their accumulation.

Materials and methods

Bacterial strain

Lactobacillus hilgardii ISE 5211 (http://www.colmia.it CRA-ENO3100), isolated from a histamine contaminated Italian red wine and belonging to the collection of Centro

di Ricerca per l'Enologia C.R.A. (Asti, Italy), was kindly supplied by Dr. Emilia Garcia Moruno. It was maintained in the MRS medium (Lactobacilli MRS broth, Difco) supplemented with 20% glycerol at −24°C.

Culture conditions

Bacteria cultures were propagated in two steps: 1 ml of frozen bacteria was inoculated in a 100-ml pre-culture (MRS medium), and, when the middle of the exponential growth phase was reached, an adequate volume of this pre-culture was inoculated in a 250 ml culture ($OD t_0 = 0.1$) at 30°C, without shaking. The pH of the medium was adjusted to 5.5 before inoculation. Bacterial growth was monitored by 600 nm optical density (OD_{600}) measurement.

The *L. hilgardii* ISE 5211 was grown in control conditions (MRS medium, pH 5.5), and in the same medium supplemented with histidine (4 g/L, MRS + His), with arginine (4 g/L, MRS + Arg) or malate (3 g/L, MRS + Mal). This strain was also grown in an MRS medium supplemented with histidine (4 g/L) plus arginine (4 g/L) (MRS + His + Arg) or supplemented with histidine (4 g/L) plus malate (3 g/L) (MRS + His + Mal). Two biological replicates of each growth condition were performed for proteomic analysis.

Genetic analyses

The *L. hilgardii* ISE 5211 cultures (2 ml) were centrifuged at 18,000×*g* for 2 min and the pellet was re-suspended in 500 µl of a Tris–EDTA buffer. Genomic DNA extraction was carried out as described by Vaquero et al. (2004). Plasmidic DNA was isolated according to the method described by Lucas et al. (2005).

The PCR reaction was performed according to Coton and Coton (2005) with the HDC1/HDC2 and HDC3/HDC4 primer pairs, but with a modification of the annealing temperatures, which were selected at 54 and 52°C, respectively. The PCR products were analyzed by agarose (2% w/v) gel electrophoresis.

Preparation of protein extracts

Equivalent amounts of cells (40 g dry weight for each experiment) were treated in each protein preparation. The cells were harvested in the middle of the exponential phase, the biomass was collected by centrifugation and washed in 40 ml 0.85% NaCl, as previously described (Pessione et al. 2005). The pellets were resuspended in 3 ml of 1 mM EDTA, 50 mM Tris–HCl pH 7.3, supplemented with 10 µl/ml of Nuclease mix (GE Healthcare), disrupted by sonication, and centrifuged, as previously described (Pessione et al. 2005). The samples were centrifuged (100,000×*g*, 1 h, 4°C) in a Beckman L8-60 M Ultracentrifuge (Type 60 rotor) and the supernatant was dialyzed against three volumes of bi-distilled water. The protein extracts were precipitated with methanol/chloroform, according to the Wessels and Flugge method (1984) and solubilized in a rehydration solution [7.0 M urea, 2.0 M thiourea, 4% CHAPS, 1% Triton X-100, 0.02 M Tris, 0.5% IPG buffer pH 4–7 (GE-Healthcare), 1% DTT]. The protein concentrations were evaluated using the 2D-Quant kit (GE Healthcare).

2-DE

IEF was performed as previously described (Pessione et al. 2005). The proteins were separated in 13 cm IPG strips (GE Healthcare) with a linear pH gradient ranging from 4 to 7. The proteins derived from 2 mg of biomass (dry weight), corresponding to a range of 250–400 µg (depending on growth condition), were loaded onto each IPG strip. IEF was performed using the IPGphor system (GE Healthcare) at 20°C with 54,000 Vhrs after 7 h of rehydration. After IEF, the strips were incubated at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris–HCl, pH 8.6 fortified with 2% w/v DTT for 15 min and subsequently for another 15 min in the same buffer supplemented with 4.5% w/v iodoacetamide. They were then sealed at the top of the 1.0 mm vertical second dimension gels as previously described (Giuffrida et al. 2001). SDS-PAGE was carried out on each sample on 9% T, 3.3% C acrylamide (Biorad) homogeneous gels. The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. The running conditions were 11°C, 600 V constant voltage, 20 mA/gel, 60 W for 15 min and 11°C, 600 V constant voltage, 40 mA/gel, 80 W for about 3 h. The molecular weight markers were from the Low Mr Electrophoresis Calibration Kit (GE-Healthcare). The gels were automatically stained using the Processor Plus (GE Healthcare) with a freshly prepared Neuhoff stain (Colloidal Comassie Brilliant Blue; Neuhoff et al. 1988) and they were dried in a GD 2000 Vacuum Gel Drier System (GE-Healthcare).

Image analysis

The 2-DE gels were digitized with the Personal Densitometer SI (GE-Healthcare). Image analysis was performed with the Progenesis PG 200 software (Nonlinear Dynamics). Spots detection was performed using the detection 2005 Algorithm made by Nonlinear Dynamics.

Statistical analysis

Four replicates were performed for each 2-DE gel. Spot intensities were measured via both absolute and normalized

spot volumes; when different spots were identified as being the same protein, the total volume of the isoforms was calculated.

The spots were statistically analyzed by one-way ANOVA, followed by Tukey's HSD post hoc test (SPSS software version 10). Moreover data were analyzed using Levene's test to verify the homogeneity of variance across samples. The mean values were considered significantly different when $P < 0.05$.

Protein identification

The protein spots were excised from the dried gels and rehydrated with MilliQ water. They were washed twice with 50% v/v ACN in a 25 mM NH_4CO_3, once in 100% v/v ACN and vacuum-dried. The proteins were in-gel digested with sequencing-grade, modified porcine trypsin (Promega, Madison, WI, USA) and added on an MALDI target plate as described by Hewitson and co-workers (2008).

Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of *m/z* 800–4,000 and monoisotopic masses were obtained from the centroid of raw, unsmoothed data. The mass spectra were internally calibrated using the tryptic auto-proteolysis products at *m/z* 842.509 and 2211.104. Mass tolerance was set to 100 ppm.

CID-MS/MS was performed on the 20 strongest peaks with a signal-to-noise greater than 40. For CID-MS/MS, a source 1 collision energy of 1 kV was used, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. Default calibration was used for the MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky–Golay with three points across a peak and a polynomial order 4); the peak detection used a minimum *S/N* of 5, a local noise window of 50 *m/z*, and minimum peak width of 2.9 bins. *S/N* 20 and 30 filters were used to generate peak lists from the MS and MS/MS spectra, respectively.

The mass spectral data from the protein spots were submitted to a database search using a locally running copy of the Mascot programme (Matrix Science Ltd, version 2.1). Batch-acquired MS/MS data were submitted to an MS/MS ion search through the Applied Biosystem GPS explorer software interface (version 3.6) to Mascot. The search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and an MS/MS tolerance of 0.1 Da. The spectra were searched against the NCBI non-redundant protein database. The significance threshold was set at $P < 0.05$, and identification required that each protein contained at least one peptide with an expected value <0.05.

De novo sequencing was performed as described by Shevchenko et al. (2001). Briefly, to include only the 50 strongest peaks, all the MS/MS spectra were filtered using FlexAnalysis (Bruker). The *De novo* sequences were computationally derived, in batch mode, for each MS/MS spectrum and combined for each spot before submitting them to http://genetics.bwh.harvard.edu/msblast/index.html for a BLAST search against the NCBI database.

Results

Histidine decarboxylase (HDC) gene detection

The presence of the HDC determinants in the DNA of *L. hilgardii* ISE 5211 was detected through gene amplification on both the total and plasmidic DNA: both reactions, using either the total or plasmid DNA with either of the primer pairs, were positive, indicating that a gene encoding an HDC was present on a plasmid in *L. hilgardii* ISE 5211 (Fig. 1).

Proteomic analyses

A comparative proteomic analysis was performed to detect any reciprocal influences between amino acid decarboxylation and the ADI pathway, and amino acid decarboxylation and malo-lactic fermentation. Six different culture conditions were considered in order to detect the highest number of differentially expressed proteins: (1) MRS medium (control condition); (2) MRS + His; (3) MRS + Arg; (4) MRS + Mal; (5) MRS + His + Arg, (6) MRS + His + Mal. Bacterial cells were harvested, for each growth

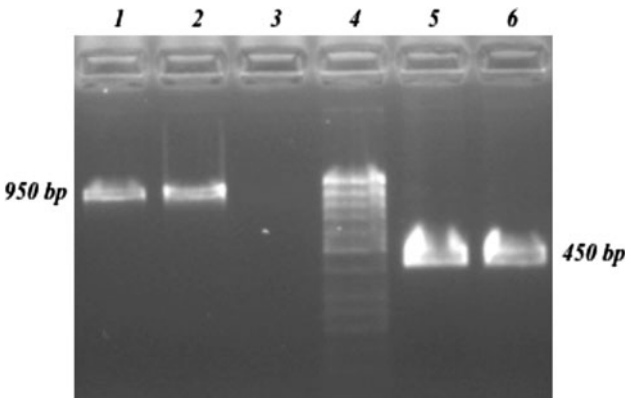

Fig. 1 Agarose gel electrophoresis (2.0%) of the PCR products obtained with HDC1/HDC2 (product size 950 bp; *lanes 1–2*) and HDC3/HDC4 (product size 450 bp) primers (*lanes 5–6*). *Lanes 1*, *5* total DNA; *lanes 2*, *6* plasmidic DNA; *lane 3* negative control; *lane 4* marker

condition, in the middle exponential phase (Fig. 2), which corresponds to the maximum consumption rate of histidine (via decarboxylative pathway), malate (via malo-lactic fermentation) and arginine (via ADI-pathway), as demonstrated in a previous work (Mazzoli et al. 2008). The total protein extracts were separated on pI 4–7 L gels: a reference map (representative gel characterized by the maximum number of spots) for each growth condition is shown in Fig. 3.

The image analysis results were submitted to statistical tests which revealed 24 differentially expressed spots on a total of 334 spots present in reference gel: 20 of these were identified by MALDI TOF/TOF and/or *De novo* sequencing (Supplementary Table). The average absolute volumes of each identified spot and their variations in each growth condition considered are shown in Table 1. A graphic representation of the most interesting protein expression profiles is given in Fig. 4. The total protein volume of the spots identified as the same protein (spots 302, 306, 311, 378 corresponding to HDC; spots 122, 123,126 identified as the ADI; 178, 184, 382 corresponding to OTCase; 89 and 550 identified as the Hystidyl-tRNA synthetase) was also presented with its corresponding standard error of the mean (SEM; Fig. 4).

The identified proteins can be divided into six functional groups: (1) amino acid decarboxylases, (2) ADI pathway enzymes, (3) malo-lactic fermentation enzymes, (4) amino acid metabolism enzymes, (5) sugar metabolism enzymes and (6) stress proteins (Table 1).

Amino acid decarboxylases

Histidine decarboxylase (spots 302, 306, 311, 378; Figs. 3, 4) was detected in low amounts in the cultures not supplemented with the precursor amino acid (MRS, MRS + Arg, MRS + Mal) suggesting that this enzyme is constitutively expressed in *L. hilgardii* ISE 5211. When the cultures were supplemented with histidine (MRS + His), a 4-fold increase in the HDC concentration was observed compared to the control conditions (MRS). When arginine was present in the medium together with histidine (MRS + His + Arg), the HDC concentration was only 2-fold higher than the "basal" level measured in the control condition (MRS), suggesting a possible interference of arginine with histidine decarboxylase biosynthesis. Conversely, malate did not affect HDC expression: the HDC concentration detected in MRS + His + Mal was the same as that measured in the MRS + His cultures.

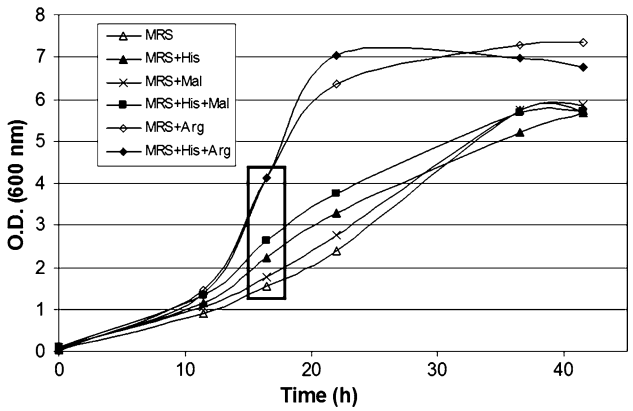

Fig. 2 *Lactobacillus hilgardii* ISE 5211 growth curves in the MRS medium, MRS + His, MRS + Mal, MRS + His + Mal, MRS + Arg, MRS + His + Arg. The highlighted area (*rectangle*) shows the harvesting time for the proteomic analysis

ADI-pathway enzymes

All the ADI pathway enzymes (ADI, spots 122,123,128; OTCase, spots 178, 184, 382; CK, spot 217; Figs. 3, 4) were up-regulated in the cultures supplemented with arginine (MRS + Arg, MRS + His + Arg). The ADI and OTC amounts were, respectively, 4- and 3-fold more expressed in the MRS + Arg and MRS + His + Arg cultures than in the control condition (MRS), while CK was, respectively, 3- and 2.5-fold more abundant in the same conditions. However, the same proteins were also detected in lower amounts in the cultures where arginine was not added (MRS, MRS + His, MRS + Mal, MRS + Mal + His): a certain level of constitutive expression has thus been proved, as described above for HDC. The constitutive expression level of the first two enzymes of the route (ADI and OTC), however, was reduced in MRS + His (4- and 2-fold for ADI and OTC, respectively), while malate (the MRS + Mal and MRS + Mal + His cultures) did not affect the basal expression of any of the enzymes of the route. Interestingly, in the cultures supplemented with both histidine and arginine (MRS + His + Arg), only the first enzyme of the pathway (ADI) was down-regulated, probably because of the opposite effects of arginine, which triggers activation, and histidine, which causes repression, in a similar way to what was observed in MRS + His + Arg for HDC expression.

Malo-lactic fermentation

The malo-lactic enzyme (MLE, spot 58; Figs. 3, 4) was not detected in the cultures where malate was not present (MRS, MRS + His, MRS + Arg, MRS + His + Arg), while it was found in large amounts in cultures supplemented with malate (MRS + Mal, MRS + His + Mal). This indicates its inducible expression differently from HDC and ADI pathway genes, constitutively transcribed.

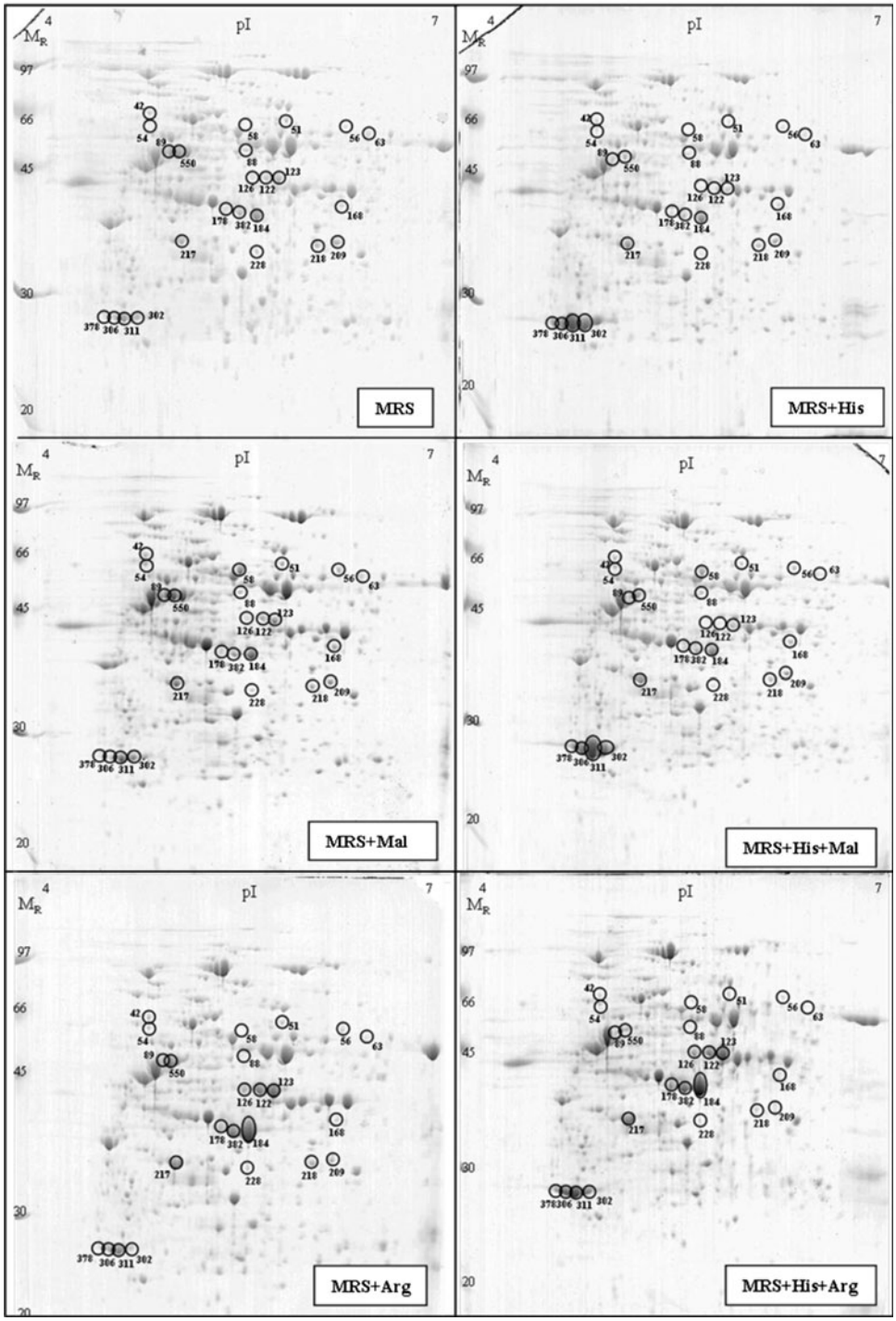

Fig. 3 Two-dimensional reference gels (IPG strip 4–7 13 cm, 9.0%T) of *L. hilgardii* ISE 5211 grown in MRS, MRS + His, MRS + Mal, MRS + His + Mal, MRS + Arg, MRS + His + Arg. Identified spots are indicated with different numbers and *circled*

Amino acid metabolism enzymes

Histidyl-tRNA synthetase (spot 89, 550; Figs. 3, 4) was down-regulated in the presence of histidine: it was 6-, 8- and 10-fold less abundant in the MRS + His + Mal, MRS + His, and MRS + His + Arg media, respectively, compared to the control conditions (MRS).

Glutamyl-tRNA synthetase (GluRS, spot 56; Figs. 3, 4) was also down-regulated in the presence of histidine although to a lesser extent (4.5-fold less expressed in MRS + His than in MRS) compared to hystidyl-tRNA synthetase. Furthermore, it was slightly but significantly up-regulated by malate (its level was 2- and 3-fold higher in the MRS + His + Mal and MRS + Mal cultures,

Table 1 Quantification of identified spots: absolute volumes (mean value) and their variations (SEM) among different culture conditions

	Spot no.	Identified protein	Absolute spot volume (mean ± SEM)					
			MRS	MRS + His	MRS + Mal	MRS + His + Mal	MRS + Arg	MRS + His + Arg
Amino acid decarboxylases	302	HDC	495.52 ± 54.78	2,344.55 ± 413.65	423.67 ± 140.96	2,311.84 ± 521.32	428.06 ± 119.77	1,085.08 ± 347.46
	306	HDC	439.12 ± 83.03	1,424.81 ± 46.53	383.72 ± 102.69	1,415.64 ± 204.63	471.43 ± 174.10	822.32 ± 110.14
	311	HDC	1,097.66 ± 170.45	4,077.82 ± 163.06	869.38 ± 55.13	4,840.22 ± 769.85	1,136.60 ± 91.71	2,185.52 ± 133.21
	378	HDC	116.72 ± 43.86	683.10 ± 82.42	131.15 ± 38.83	747.83 ± 187.74	115.95 ± 73.82	223.71 ± 43.82
ADI-pathway enzymes	122	ADI	310.61 ± 47.48	142.49 ± 23.26	310.82 ± 15.95	238.93 ± 51.58	1,678.98 ± 62.93	1,213.11 ± 206.96
	123	ADI	613.06 ± 91.30	157.46 ± 12.60	465.76 ± 49.35	341.94 ± 26.38	3,002.56 ± 225.07	2,224.63 ± 347.69
	126	ADI	217.58 ± 76.45	n.d.	n.d.	79.18 ± 37.26	156.89 ± 62.79	15.11 ± 11.80
	178	OTCase	n.d.	146.77 ± 46.12	n.d.	120.95 ± 7.16	576.41 ± 78.59	719.62 ± 93.43
	184	OTCase	1,664.52 ± 288.10	917.94 ± 17.26	1,255.42 ± 42.41	1,182.33 ± 75.04	6,226.65 ± 727.44	4,469.92 ± 677.13
	382	OTCase	662.44 ± 138.85	223.49 ± 26.90	382.50 ± 32.95	360.65 ± 18.27	2,018.58 ± 281.06	1,675.03 ± 257.64
	217	CK	591.51 ± 79.99	626.53 ± 64.36	579.86 ± 89.08	812.77 ± 110.76	1,896.51 ± 319.77	1,470.80 ± 196.86
Malo-lactic fermentation enzymes	58	MLE	n.d.	n.d.	606.83 ± 28.50	541.82 ± 31.55	n.d.	n.d.
Amino acid metabolism enzymes	89	HisRS	1,110.07 ± 302.99	96.74 ± 37.93	907.70 ± 141.76	463.92 ± 177.75	1,040.99 ± 283.13	n.d.
	550	HisRS	1,535.37 ± 311.90	223.42 ± 29.16	1,115.48 ± 121.72	n.d.	665.79 ± 234.10	254.28 ± 53.36
	56	GluRS	58.58 ± 3.95	13.06 ± 6.35	180.71 ± 15.00	136.04 ± 8.89	88.17 ± 18.63	69.27 ± 29.88
	88	ASS	111.75 ± 4.98	n.d.	133.67 ± 10.28	108.63 ± 7.37	n.d.	n.d.
Sugar metabolism enzyme	51	PGM	156.61 ± 17.01	21.21 ± 3.91	144.71 ± 6.32	71.95 ± 3.26	108.25 ± 18.27	63.22 ± 6.19
	218	PDH	302.49 ± 33.91	127.58 ± 25.35	226.30 ± 13.60	173.30 ± 9.41	326.41 ± 19.09	72.02 ± 36.79
Stress protein	42	GroEL	150.84 ± 20.01	49.88 ± 5.06	149.20 ± 16.20	139.05 ± 23.14	135.41 ± 26.63	33.45 ± 13.24
Not identified and unknown function proteins	228	Oxidoreductase	109.98 ± 6.47	n.d.	77.31 ± 13.61	28.53 ± 8.22	94.74 ± 25.66	n.d.
	54	Not identified	36.55 ± 13.73	24.71 ± 15.65	145.61 ± 6.32	152.36 ± 27.65	74.80 ± 21.51	64.66 ± 12.81
	63	Not identified	118.75 ± 18.31	n.d.	84.28 ± 33.90	80.29 ± 28.25	n.d.	n.d.
	168	Not identified	81.97 ± 10.65	71.22 ± 5.72	203.74 ± 11.89	174.92 ± 10.82	86.64 ± 13.06	77.61 ± 8.39
	209	Not identified	470.88 ± 66.67	269.82 ± 8.78	316.99 ± 17.37	264.54 ± 15.63	146.25 ± 73.63	101.22 ± 50.02

n.d. not detectable

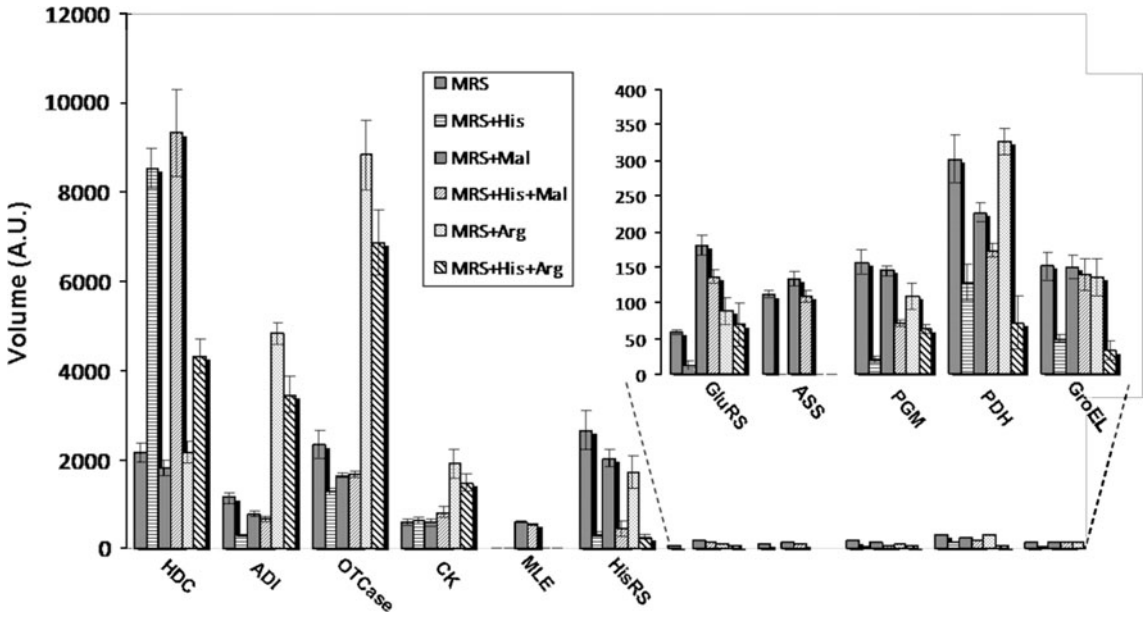

Fig. 4 Histogram of the expression profiles of the identified proteins in the different analyzed conditions. The total volumes of the isoforms and the SEM are shown

respectively, than in the control condition). Finally, arginine (MRS + Arg and MRS + His + Arg cultures) does not seem to have any effect on the expression levels of this enzyme.

The argininosuccinate synthetase (ASS, spot 88; Figs. 3, 4) was not detected in the cultures supplemented with arginine (MRS + Arg, MRS + His + Arg), while it was produced in the same amount in most of the other experimental conditions. This enzyme is the key enzyme of arginine biosynthesis: it catalyzes the conversion of citrulline, aspartate and ATP into arginino-succinate, which can be hydrolyzed into arginine and fumarate by argininosuccinase, the next enzyme in the pathway (Fig. 5a).

Sugar metabolism

Phosphoglucomutase (PGM, spot 51) and pyruvate dehydrogenase (PDH, spot 218; Figs. 3, 4), both of which are involved in energetic routes, were down-regulated in the cultures supplemented with histidine (7-fold for PGM and 2.5-fold for PDH in MRS + His, 2-fold for PGM and 1.5-fold for PDH in MRS + His + Mal, 2.5- fold for PGM and 4-fold for PDH in MRS + His + Arg). PGM catalyzes the conversion of glucose-1P into glucose-6P which then enters the glycolysis and/or pentose phosphate pathway, while PDH is involved in the conversion of pyruvate into acetyl-CoA; this latter can undergo further transesterification to acetyl-P, which can be used by acetate kinase to generate ATP (Fig. 5b). The down-regulation of these two enzymes in the histidine supplemented cultures (MRS + His, MRS + His + Mal, MRS + His + Arg) could therefore be interpreted as an inhibition of energy supplying routes when bacteria can obtain enough energy from histidine decarboxylation. However, the same is not true for the other two energy generating routes considered above (ADI pathway and MLF).

Stress proteins

The 60 kDa heat shock protein (GroEL, spot 42; Figs. 3, 4) was down-regulated in the cultures supplemented with histidine (3-fold in MRS + His and 4.5-fold in MRS + His + Arg compared to MRS).

Discussion

The present proteomic investigations offer clear evidence that the histidine decarboxylation pathway in *L. hilgardii* ISE 5211 is modulated at a biosynthetic level by both histidine (activation) and arginine (attenuation), while malate displays no regulative action on HDC expression. The same inducing effect of histidine had been observed on the HDC operon in a previous proteomic investigation on two *Lactobacillus* strains (30a and w53) (Pessione et al. 2005) and it had also been demonstrated through transcript analyses by Landete et al. (2006) on *L. hilgardii*, *O. oeni*, and *P. parvulus*. Induction/enhancement of the transcription of their own specific decarboxylases has also been referred for other amino acids such as tyrosine (Pessione et al. 2009), lysine and ornithine (Guerrini et al. 2002).

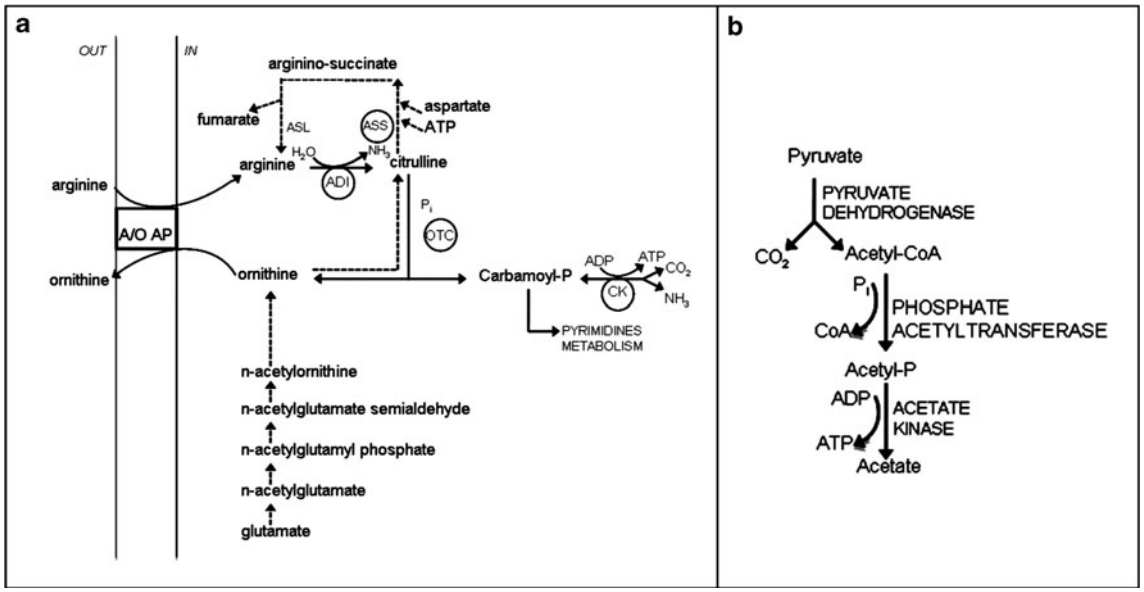

Fig. 5 **a** Arginine biosynthesis (*discontinuous arrow*) and ADI pathway (*continuous arrow*). The enzymes of the pathways that are identified are *circled*. **b** Pyruvate metabolism via pyruvate dehydrogenase

Furthermore, in a previous work on *L. hilgardii* ISE 5211 (Mazzoli et al. 2008), the kinetics of histamine accumulation appeared to be slightly delayed when histidine and arginine were both present in the medium: we have hypothesized a biosynthetic regulation, exerted by arginine, on HDC transcription on the basis of SDS-PAGE data. The present proteomic experiments have allowed us to define, with statistical significance, this modulation as a biosynthetic repression: to the authors' knowledge, this is the first evidence that arginine down-regulates HDC biosynthesis.

The ineffectiveness of malate on HDC expression and the ineffectiveness of histidine on MLE biosynthesis are also in agreement with previously reported data based on growth kinetics studies (Mazzoli et al. 2008) and indicate that MLF and histidine decarboxylation are parallel routes that do not interfere with each other. However, this result does not agree with the data reported by Landete et al. (2006) who found, by means of Northern blotting transcript analyses, that malate can inhibit HDC gene expression in *L. hilgardii*. It is important to notice that mRNA and the protein level of the same gene product may differ because of the modulation of the translation efficiency: it is thus possible that the lower number of HDC mRNA copies observed in the presence of malate in *L. hilgardii* by Landete et al. (2006) was translated more efficiently and this resulted in the same HDC level as cultures not supplemented with malate. Alternatively, we cannot exclude that different strains can differ as far as gene regulation mechanisms are concerned. Finally, in agreement with our evidence on HDC, Mangani et al. (2005) stated that, in *O. oeni*, the decarboxylation of ornithine to putrescine proceeds parallel with MLF without any reciprocal inhibition.

The arginine activation exerted over ADI was previously referred by other authors, as either induction (Manca de Nadra et al. 1986) or enhancement (Tonon et al. 2001; De Angelis et al. 2002) of its enzyme constitutive expression level in LAB isolated from both wine and cheese.

The histidine inhibition of ADI and OTC is in agreement with what has been observed in *L. lactis* NCDO 2118 (Mazzoli et al. 2010) using glutamate-supplemented cultures. In this strain, the glutamate-stimulated condition exerted an inhibition on the ADI operon at both the transcript and protein level, in a similar way to what has been observed in the present investigation on *L. hilgardii* ISE 5211, grown on histidine. On the contrary, no interference of tyrosine on ADI enzymes biosynthesis was detected in *E. faecalis* (Pessione et al. 2009).

Considering the energy-supplying physiological function of malate and histidine decarboxylation versus arginine deimination, it is possible to state that the two PMF generating routes can be simultaneously activated, while arginine deimination and histidine decarboxylation seem to reciprocally compete. Although quantifying the energy gain of a proton gradient is more complex than quantifying produced ATP moles, the down-regulation of sugar metabolism, highlighted by proteomics, suggests that the PMF obtained by histidine decarboxylation is better able to solve the energy requirements than ADI. Nevertheless, arginine, via ADI, supports a higher growth rate (μ) and a higher biomass yield than histidine (Fig. 2). This can be explained considering the important role played by

arginine in pH buffering: since arginine deimination gives rise to NH_3 ($pK_a = 9.3$), it is better able to control excess acidification (due to lactic acid accumulation during lactic fermentation) than histamine ($pK_a = 5.1$).

Some considerations can be made concerning the observed down-regulation of some amino acid metabolism enzymes. By sequencing the *hdc* locus of *L. hilgardii* IOEB 0006, Lucas et al. (2005) demonstrated the existence of a cluster of four genes located on a plasmid (the same location that has been demonstrated for HDC gene of our strain, Fig. 1). This cluster encodes, for a histidine decarboxylase (HDC), an integral membrane transporter that drives substrate/product exchange (HdcP), a protein with an unknown function and a hystidyl-tRNA synthetase (HisRS). In general, aminoacyl-tRNA synthetases play an essential catalytic role in protein biosynthesis, but the presence of their genetic determinants in the amino acid decarboxylation clusters may indicate additional physiological roles (Fernandez et al. 2004). In this context, Martin et al. (2005) demonstrated, in *L. buchneri*, that the starting codon upstream from the region of HisRS contains a putative promoter and a leader region with the sequence features of a tRNA-mediated anti-termination system. They suggested that in the absence of the corresponding amino acid (e.g. histidine for HisRS) the uncharged tRNA destabilizes the transcription terminator structure generated by the leader region thus allowing transcription. Conversely, when histidine or other amino acids are present, histidyl-tRNA (or alternatively any aa-tRNA) cannot interact with the terminator structure and thus represses transcription. Since, in the strain under study (*L. hilgardii* ISE 5211), the hystidyl-tRNA synthetase was down-regulated in the cultures supplemented with histidine, a repression on the *hisRS* promoter by a tRNA-mediated anti-termination system is proposed.

The down-regulation of ASS was expected: the arginine biosynthetic pathway is closely connected to the ADI route. When arginine is available in a medium (MRS + Arg, MRS + His + Arg) bacteria take it up and channel it towards the ADI pathway, while ASS is no longer necessary and the whole arginine biosynthetic pathway is down-regulated (inhibition by the final metabolic product; Fig. 5a).

Finally, histidine seems to protect *L. hilgardii* ISE 5211 from stress: as already demonstrated by other authors, GroEL is over-produced in bacteria during stress conditions (Desmond et al. 2004). This could suggest that the energy obtained from HDC activity can aid bacteria to attenuate stress while the other energy generating pathways (ADI and MLF) seem to be ineffective in counterbalancing this stress. A protective effect of amino acids against stress was previously observed in *L. lactis* NCDO 2118 grown in high glutamate concentrations: stress related proteins such as CtsR, superoxide dismutase and ClpP protease, were down-regulated (Mazzoli et al. 2010). On the contrary, the amino acids tyrosine and phenylalanine can exert an inducing action on stress proteins such as Dna J and Gls 24, as demonstrated in a previous proteomic investigation (Pessione et al. 2009).

From an applicative point of view, in spite of the possible negative effects of histamine on the vascular and immune systems, histamine accumulation in wine is preferable to NH_3 since the latter is extremely toxic. Furthermore, during the ADI route, carbamoyl-phosphate, derived from citrulline conversion into ornithine, can react with ethanol and gives rise to ethylcarbamate, which is carcinogenic. Therefore, the reciprocal competition between ADI and HDC could result in a prevention of more severe damage.

These metabolic pathways are generally triggered by the energy requirements of the LAB used for MLF: therefore, for oenological applications, the choice of a strain bearing the genetic determinants for MLF but not for HDC or ADI is very important, even though a certain degree of modulation of their expression exists. Last but not least, in the strain under study, the genetic determinants for HDC are located on a plasmid as was previously found by Lucas et al. (2005) for *L. hilgardii* 0006: considering the frequency of genetic recombination in bacteria (by phage transduction, transformation and conjugation), this finding is not favorable since it enhances the possibility of a spread of the ability to decarboxylate histidine, among the bacteria that colonize the wine ecological niche, and this results in higher health risks.

Acknowledgments This research has been carried out within CESQTA and financed by grants from the Regione Piemonte (CIPE 2004, Progetto di Ricerca Sanitaria Finalizzata 2004). The authors are grateful to Emilia Garcia-Moruno who kindly supplied the *L. hilgardii* ISE 5211 strain.

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