International Journal of Food Microbiology 188 (2014) 99–107

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

α-Acetolactate synthase of *Lactococcus lactis* contributes to pH homeostasis in acid stress conditions

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ARTICLE INFO

Article history:
Received 4 February 2014
Received in revised form 3 June 2014
Accepted 16 July 2014
Available online 27 July 2014

Keywords:
Lactococcus lactis
Acetoin/diacetyl pathway
α-Acetolactate synthase
pH homeostasis
Pyruvate metabolism

ABSTRACT

Lactic Acid Bacteria (LAB) are recognized as safe microorganisms with the capacity to improve the quality of dairy products. When the LAB *Lactococcus lactis* is employed as starter for the production of fermented foods, high quantities of important aroma compounds such as diacetyl are generated by means of the diacetyl/acetoin pathway. Our previous results obtained with *L. lactis* strains report that this pathway is activated under acidic conditions. In this study, we describe the metabolism of pyruvate, a diacetyl/acetoin precursor, and its contribution to pH homeostasis in this microorganism. *L lactis* strain IL1403 is able to cometabolize pyruvate and glucose at low pH, producing lactate, acetate as well as diacetyl/acetoin compounds. In contrast, the *als* defective strain, which is incapable of producing C4 compounds, appeared sensitive to pyruvate under acidic conditions rendering it unable to grow. Accordingly, the *als*-mutant strain showed a simultaneous inability to alkalinize internal and external media. These results demonstrate that the decarboxylation reactions associated to the diacetyl/acetoin pathway represent a competitive advantage in a condition of intracellular pyruvate accumulation during growth at low pH. Interestingly, a genomic comparative analysis shows that this pathway has been conserved in *L. lactis* during the domestication of different strains. Also, our analysis shows that the recent acquisition of the *cit* cluster required for citrate metabolism, which contributes to diacetyl/acetoin production as well, is the specific feature of the biovar. diacetylactis. In this regard, we present for first time genetic evidence supporting the proposal made by Passerini et al. (2013) who postulated that the expression "biovar. citrate" should be more appropriate to define this specific industrial strain.

1. Introduction

One of the most ancient practices to improve food quality involves fermentation by lactic acid bacteria (LAB). Among others, *Lactococcus lactis* is extensively used in dairy food fermentation. This microorganism is able to metabolize sugars and acidify milk, preventing growth of unwanted bacteria. In addition, *L. lactis* generates different C4 aroma compounds (acetoin, diacetyl and 2,3-butanediol) that improve the organoleptic characteristics of fermented food. Such is the case of diacetyl, which has a strong buttery flavor and is desirable at high concentration in dairy products. Based on traits important from an industrial point of view, three phenotypes are recognized for lactococcal strains: *L. lactis* (metabolizes arginine, capable of growing at high temperature and salt concentration), *Lactococcus cremoris* (defective in arginine metabolism, unable to grow at high temperature or salt concentration) and *Lactococcus diacetylactis* (metabolizes citrate). However, *L. lactis* could be phylogenetically differentiated into two subspecies *lactis* and *cremoris*, with the biovar. diacetylactis grouping within the *lactis* subspecies (Kelly et al., 2011). The biovar. diacetylactis is one of the most important starters used in dairy manufacture due to its capacity to degrade citrate present in milk and increase the amount of diacetyl in cheese (Kelly et al., 2011).

In *L. lactis*, the diacetyl/acetoin biosynthetic route has been studied and it was determined that the α-acetolactate synthase (ALS) constitutes the first enzyme of the pathway (Snoep et al., 1992). This enzyme catalyzes the condensation of two pyruvate molecules to give α-acetolactate (AL, Fig. 1). The apparent K_m for pyruvate of ALS from *L. lactis* was found to be approximately 50 mM, with an optimum pH of 6.0 as determined in vitro for the purified enzyme (Snoep et al., 1992). ALS shows a very low affinity for pyruvate, which suggests that this enzymatic reaction is favored under conditions of intracellular accumulation of its substrate and acidic pH. These conditions are indeed those empirically determined to be optimal for the production of C4 aroma compounds during *L. lactis* fermentation: co-metabolism of sugars and secondary carbon sources, such as citrate, which increase

Abbreviations: ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.017
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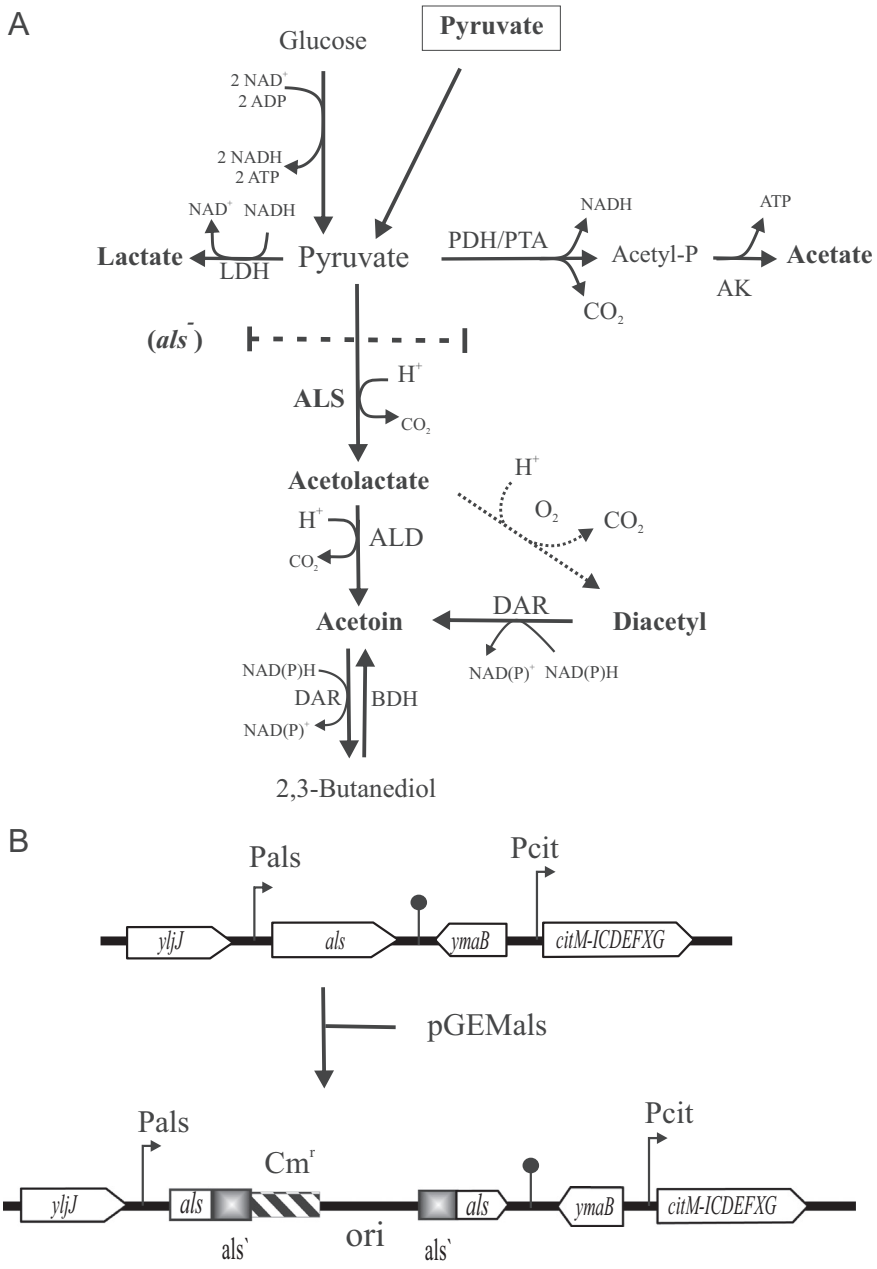

Fig. 1. A) Pyruvate metabolism in *Lactococcus lactis*. The graphic shows the enzymes involved: Lactate dehydrogenase, LDH; Pyruvate dehydrogenase, PDH; Phosphotransacetylase, PTA; Acetate kinase, AK; ALS, α-acetolactate synthase; ALD, α-acetolactate decarboxylase; DAR, diacetyl-acetoin reductase; BDH, butanediol dehydrogenase. H^+, proton scalar consumption is indicated for ALS and ALD catalytic steps. O_2, oxidative decarboxylation. B) Schematic representation of *als* inactivation by insertion of pmAls plasmid. *als′* is a central fragment of the *als* gene. Cm^r represents the chloramphenicol resistance cassette (striped lines).

internal pyruvate concentration and low external pH due to acidic by-product generation (Hugenholtz and Starrenburg, 1992). Once AL is formed, it can be enzymatically converted into acetoin or non-enzymatically into diacetyl by an oxidative decarboxylation step (Fig. 1). Acetoin is synthesized enzymatically by decarboxylation of AL or by reduction of diacetyl in reactions catalyzed respectively by α-acetolactate decarboxylase (ALD) (Phalip et al., 1994) and diacetyl-acetoin reductase (DAR), which is also capable of subsequently reducing acetoin to 2,3-butanediol (Rattray et al., 2000). It has been shown that the DAR activity is strain-dependent in LAB (Cogan et al., 1981). In addition, acetoin can be produced by a chemical non-oxidative decarboxylation of AL, since this compound is unstable at acidic pH (Marugg et al., 1994). Many microorganisms expressing a functional C4 pathway also express a butanediol dehydrogenase (BDH). This enzyme is involved in regeneration of acetoin from 2,3-butanediol requiring NAD^+ as cofactor (Xiao and Xu, 2007).

In *L. lactis* subsp. *lactis* biovar. diacetylactis the generation of compounds that improve aroma is linked to citrate metabolism. In this sense, numerous studies point to the elucidation of the route and the transcriptional regulation of genes involved in this pathway. In *L. diacetylactis* CRL264, this metabolism has been extensively characterized by our group during recent years (García-Quintans et al., 1998, 2008; López de Felipe et al., 1996; Magni et al., 1994, 1996, 1999; Martin et al., 2004, 2005). We demonstrated that the operons involved in citrate transport (*citQRP* operon) and its sequential conversion to pyruvate by the citrate lyase and a soluble oxaloacetate decarboxylase (*citM-ICDEFXG*) are induced at low pH (García-Quintans et al., 1998; Martin et al., 2004). In addition, we described that the genes involved

in C4 compound production (*als*, coding for the ALS; *aldB* and *aldC* coding for two ALD isoforms and the *butBA* operon, encoding a BDH and a DAR, respectively) are specifically induced under the same conditions (Garcia-Quintans et al., 2008). Consistently, a proteomic analysis under acidic conditions on the same strain revealed an increase in expression for ALS, whereas expression levels for LDH (lactate dehydrogenase) and PDH subunits (pyruvate dehydrogenase complex) remained constant (Garcia-Quintans et al., 2008). Thus, our previous results suggest that excess pyruvate is deviated towards the production of neutral compounds. Synthesis of these metabolites was relevant in maintaining internal pH homeostasis at low pH.

In addition, other LAB are capable of switching their metabolism to the production of neutral compounds with the aim of avoiding lethal acidification (Tsau et al., 1992). For example, the *alsSD* operon contributes to the mechanisms of pH resistance in *Enterococcus faecalis*, which is capable of utilizing pyruvate as carbon and energy source (Repizo et al., 2011). In this sense, it is worth noting that some LAB such as *Oenococcus oeni* and *Leuconostoc mesenteroides* could also use pyruvate as an external electron acceptor (Wagner et al., 2005), even though a pyruvate transporter has not yet been described in this group of bacteria.

The fully-sequenced *L. lactis* strain IL1403 derives from *L. lactis* subspecies *lactis* biovar. diacetylactis strain IL594, which was isolated from a starter culture for cheese and it harbors, among others, a plasmid carrying a citrate transporter gene (Górecki et al., 2011). Since strain IL1403 has been cured of plasmids and does not encode a chromosomal citrate transporter, this allowed us to study the contribution of the C4 route without interference of citrate metabolism. For this strain, the genetic background associated to the C4 pathway and its contribution to pH homeostasis has not been explored before. In this work, we demonstrate that a strain with a disruption of the *als* gene does not produce C4 compounds (diacetyl, acetoin or 2,3-butanediol) and shows an increase towards the toxicity caused by pyruvate addition under acidic conditions as well. Moreover, our genome-scale analysis indicates that this capacity to control internal pH by production of flavor compounds was present in *L. lactis* previous to its differentiation into subspecies. All these results confirm the essential role of ALS in avoiding intracellular acidification due to the accumulation of pyruvate during sugar fermentation at low pH.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lactococcal strains and plasmids used in the present study are shown in Table 1. Cultures of *L. lactis* were routinely grown at 30 °C without shaking in 100-ml sealed bottles containing 20 ml of lactose-free M17 medium, supplemented with 25 mM glucose (M17G). Chloramphenicol (10 μg ml^{-1}) was added when appropriate. Overnight cultures prepared in this way were used to inoculate fresh M17 adjusted to different initial pH values as indicated. Inoculums were diluted to an initial OD_{600} of 0.08 for cultures adjusted to pH 7.0, 0.10 for pH 5.5 and 0.12 for pH 4.5. The medium was supplemented with 11 mM of glucose (M17G), 25 mM of pyruvate (M17P) or both carbon sources (M17GP) at the specified concentrations.

E. coli strains were routinely grown aerobically at 37 °C in LB and transformed as previously described (Sambrook et al., 1989). Growth was monitored by measuring OD_{600} in a Beckman DU640 spectrophotometer. Aerobic growth was achieved by gyratory shaking at 250 r.p.m. Ampicillin (100 μg ml^{-1}) was included in the medium in order to select cells harboring the different plasmids. Plates supplemented with 20 μg/ml X-Gal and 0.15 mM IPTG were used to identify recombinant plasmids with DNA insertions that impaired ß-galactosidase activity in a DH5α strain.

Table 1
Strains and plasmids used in this study.

Designation	Relevant characteristics	Reference or source
Strains		
L. lactis		
IL1403	*L. lactis* subspecies *lactis* biovar. diacetylactis plasmid-free strain derived from IL594 strain	Bolotin et al. (2001)
lLGR1	IL1403 isogenic derivative *als* mutant	This study
E. coli		
DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17, used as an intermediate host for cloning	Hanahan (1983)
Plasmids		
pGEMT-easy		Promega
pGEMals	pGEMT derivative carrying *als* gene 487 bp-internal fragment and a chloramphenicol resistant cassette	This study
pUC18Cm	pUC18 derivative carrying a chloramphenicol resistant cassette	Laboratory stock

2.2. Interruption of als gene

In order to inactivate the *als* gene from *L. lactis* IL1403, a central fragment of this gene (als′; 487 bp) was PCR-amplified using the Alsmut1 (GGGGAATTCATCTATTGATGAC, EcoRI) and Alsmut2 (GCCATCGA GATAAGCTTTTGTTCC, HindIII) primers, and chromosomal DNA from strain IL1403 as template. The PCR product was restricted with HindIII. In parallel, plasmid pUC18Cm (Table 1), carrying a chloramphenicol-resistance cassette (Cm^r), was treated with HindIII and BamHI in order to release this cassette from the vector. Next, als′ and Cm^r were ligated through the HindIII site, and the resulting fragment was used as template for a new PCR assay using again Alsmut1 primer combined with Cm2 primer (TAAGACTGGATCCTGGAGCTGTAAT, BamHI), which anneals to the 3′ side of Cm^r. The amplicon thus obtained was cloned by ligation through its A-overhangs into the commercial vector pGemT-easy, originating the pmAls plasmid (Table 1), which was electroporated into *L. lactis* IL1403 (chloramphenicol-sensitive strain). Given that this vector is not replicative in *L. lactis*, chloramphenicol-resistant transformants should carry an *als* inactivated gene. Insertional events were checked by PCR and Southern Blot as described by Sambrook et al. (1989) (Fig. S1). The double-stranded probes hybridizing with *als* were labeled by incorporation of [α-^{32}P] dATP in the PCR amplification mix using oligonucleotides Alsmut1 and Alsmut2. The resultant *als*-deficient strain was named ILGR1 (Table 1).

2.3. Quantification of C4 compounds

Concentration of C4 compounds (diacetyl and acetoin) was determined by an adaptation of the Fertally and Facklam method (1987). Briefly, strains were grown in M17G, M17GP or M17P medium for 24 h at 30 ° C. Supernatants of each culture (175 μl) were transferred to 96-well microplates and 25 μl of a freshly prepared solution containing 15% w/v α-naphthol and 0.5% w/v creatine in 7.5 M NaOH was added. Once the mix was prepared, the microtitre plate was loaded into the microplate reader and absorbance was recorded every 2 min at 540 nm until reaching a constant absorbance value. C4 compound concentration per well was calculated using the regression equation for the acetoin standard curve. A cell-free control in which M17 supplemented with pyruvate was incubated for 24 h at 30 °C and included as blank. Results are presented as the mean value and standard deviation corresponding to assays performed in triplicate.

2.4. Pyruvate consumption

Pyruvate concentration was determined enzymatically with a lactate dehydrogenase (LDH)-based assay. A 10 μl aliquot of the sample was added to 190 μl of 100 mM sodium phosphate buffer pH 7.5 containing NADH (final concentration 0.7 mM), BSA (final concentration

0.035%) and LDH (final concentration 4.8 U/ml). Pyruvate in the sample was measured by addition of LDH which results in the conversion of pyruvate to L-lactate at the expense of NADH, resulting in a decrease in the NADH concentration equivalent to the pyruvate concentration present in the samples. The assay was performed in 96-well microtiter plates. The decrease in NADH concentration was measured spectroscopically at 340 nm.

2.5. Loading of cells with the BCECF probe and internal pH measurements

Loading of cells with the fluorophore was performed as previously indicated (Espariz et al., 2011), with the following modifications. Cells were washed once with 50 mM potassium phosphate buffer (KPi) pH 5.8 and loaded with the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF) as previously described (Magni et al., 1999). The suspension was incubated for 5 min at RT, then washed and resuspended in 50 mM KPi pH 5.8 and finally stored on ice until used. Internal pH changes were followed in the same buffer using the protocol reported by Espariz et al. (2011). Excitation wavelength was 503 nm and fluorescent emission was recorded at 525 nm (slit widths were 4 and 16 nm, respectively). Cytoplasmic pH was determined from the fluorescence signal as previously described (Magni et al., 1999).

2.6. Preparation of resting-cell suspensions

Cells grown in M17G up to the mid-exponential phase ($OD_{600} = 0.6$) were harvested by centrifugation for 10 min at 4500 g. Pellets were washed twice with 50 mM KPi buffer pH 5.8, and finally resuspended in the same buffer. Assays were performed in a total volume of 1.5 ml with resting cells ($OD_{600} = 1.5$) at pH 4.5 or 5.5. Resting cells were incubated at 30 °C without agitation for 120 min. At t = 0 min, pyruvate, glucose or pyruvate plus glucose were added at a final concentration of 20 mM pyruvate and 5 mM glucose. Cell suspension samples (100 μl) were taken at 0 and 120 min and immediately centrifuged for 0.5 min at 1200 g. Supernatants were stored on ice until further HPLC analysis (see below).

2.7. Analytical methods: HPLC analysis

Products of pyruvate metabolism were determined by loading a 20 μl aliquot of the supernatant on an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, CA) operated at 30 °C in isocratic mode using 5 mM H_2SO_4 as the mobile phase and a flow rate of 0.8 ml/min. Quantification of acetoin, lactate, acetate and pyruvate was based on calibration curves using standards.

2.8. L. lactis genome-scale analysis

Lactococcal genomic sequences were obtained from ftp NCBI. *L. lactis* subsp. *lactis* strains used in this analysis were as follows: the industrial starter IL1403 (NC_002662.1) (Bolotin et al., 2001); the plant-derived KF147 (NC_013656.1) (Siezen et al., 2010); industrial isolate IO-1 (NC_020450.1) (Kato et al., 2012) and the vaginal human probiotic CV56 (NC_017486.1) (Gao et al., 2011). Moreover, *L. lactis* subsp *cremoris* strains included were: SK11 (NC_008527.1) (Siezen et al., 2005), MG1363 (NC_009004.1) (Wegmann et al., 2007), A76 (NC_017492.1) (Bolotin et al., 2012) and UC509.9 (NC_019435.1) (Ainsworth et al., 2013), all used in the dairy industry. Comparative analysis was performed using the RAST Server (Rapid Annotations using Subsytems Technology, version 4.0) (Aziz et al., 2008) and Mauve software (version 2.3.1) (Darling et al., 2004).

3. Results

3.1. The als gene is required for pyruvate utilization at low pH in L. lactis

To analyze the specific contribution of the C4 biosynthetic pathway to the acidic response, a *L. lactis* IL1403 strain defective for the *als* gene was constructed. The gene was interrupted by single crossover chromosomal integration of the suicide plasmid pmAls, generating an *als* mutant strain (ILGR1, Table 1, Fig. S1).

Levels of C4 compounds were determined by using the Voges Proskauer reaction in culture supernatants of lactococcal strains. Thus, *L. lactis* strains were cultivated in M17 medium with or without the addition of either 11 mM glucose (M17G), 25 mM pyruvate (M17P) or both carbon sources (M17GP). An aliquot of each culture supernatant was taken after 24 h and C4 compound levels were determined. No C4 compounds were detected when *L. lactis* IL403 (wild-type strain) was set up at initial pH 7.0, under any condition (M17, M17G, M17P or M17GP, data not shown). However, when the initial pH medium was adjusted to 4.5 or 5.5 units, we observed that *L. lactis* IL1403 synthesized C4 compounds while the *als*-defective ILGR1 strain was unable to produce them (Table 2). Remarkably, *L. lactis* IL1403 produced the higher levels of C4 compounds when glucose and pyruvate were both present in the medium.

Based on these results, growth of the *L. lactis* wild type and *als* mutant strains was compared when cultured in M17, M17G, M17P or M17GP medium and initial pH was adjusted to values of 4.5 or 5.5 units. Batch cultures were developed over 8 h (see Materials and methods section) and growth parameters (μ_{max}: maximal growth rate, OD_{max}: maximal biomass) were determined for each condition (Table 3).

Wild-type IL1403 and the *als*-defective ILGR1 strains showed similar growth patterns and growth parameters in M17G (Fig. 2A versus 2D and Fig. 3A versus 3D, empty circle), even after observing a significant reduction in growth parameters for both strains when the initial pH was reduced from 5.5 to 4.5 (Table 3). In the same way, the effect on growth for both strains after the addition of pyruvate to the medium was evaluated. At initial pH 5.5, the growth curve for strain IL1403 was similar to that corresponding to strain ILGR1 (compare Fig. 2A and D, filled triangle). However, the effect of pyruvate on growth for the *als*-defective strain is evident when initial pH was fixed at 4.5 (Fig. 3A and D, filled triangle). In this case, the wild type strain was able to grow (ODmax = 0.34 ± 0.07 and μ = 0.23 ± 0.02) whereas the *als* mutant was completely arrested (Table 3). This defective phenotype is also marked in the ILGR1 strain when both compounds (glucose and pyruvate) were added to the medium. As shown in Figs. 2A and 3A and Table 3, under these conditions the wild type lactococcal cells showed the higher values for growth rate and ODmax whether the initial pH was fixed to 4.5 or 5.5. In order to explore the toxicity of pyruvate towards lactococcal cells grown at pH 4.5, we determined the number of viable cells on plates by serial dilution of each culture. While for the wild type strain we observed 10^9 CFU/ml, the viable cells were reduced to 50 CFU/ml in the *als* defective strain (Fig. S2). All these observations suggest that the *als* gene fulfills an important role in pyruvate utilization at low pH. Growth curves in M17 without any carbon sources were also

Table 2
C4-compound production (μM) in *L. lactis* IL1403 and *als* defective strains.[a]

	IL1403 (wt) pHi		ILGR1 (*als*′) pHi	
	5.5	4.5	5.5	4.5
Glucose	116 ± 18	109 ± 14	n.d.	n.d.
Pyruvate	145 ± 25	186 ± 26	n.d.	n.d.
Glucose + pyruvate	300 ± 41	348 ± 51	n.d.	n.d.

[a] Cells were grown in M17 medium adjusted at indicated initial pHs (pHi) over 24 h. C4 production was under the detection level for cells grown in M17 with no additions. n.d.: not detectable.

Table 3
Growth parameters and extracellular pH.

	pH_i 5.5			pH_i 4.5		
	pH_f	OD_{max}	$\mu_{max}(h^{-1})$	pH_f	OD_{max}	$\mu_{max}(h^{-1})$
L. lactis IL1403						
M17	5.5 ± 0.1	0.33 ± 0.02	0.32 ± 0.02	4.5 ± 0.1	0.29 ± 0.11	0.20 ± 0.03
Glc	4.7 ± 0.1	0.71 ± 0.09	0.48 ± 0.01	4.3 ± 0.1	0.35 ± 0.10	0.20 ± 0.01
Pyr	5.5 ± 0.1	0.37 ± 0.04	0.36 ± 0.01	5.2 ± 0.1	0.34 ± 0.07	0.23 ± 0.02
Glc + Pyr	5.4 ± 0.1	1.11 ± 0.01	0.53 ± 0.02	5.0 ± 0.1	0.86 ± 0.03	0.35 ± 0.01
L. lactis ILGR1						
M17	5.2 ± 0.2	0.33 ± 0.02	0.32 ± 0.04	4.5 ± 0.1	0.28 ± 0.10	0.21 ± 0.04
Glc	4.5 ± 0.1	0.74 ± 0.01	0.46 ± 0.01	4.3 ± 0.1	0.38 ± 0.05	0.25 ± 0.01
Pyr	5.4 ± 0.1	0.43 ± 0.09	0.36 ± 0.01	4.6 ± 0.1	0.09 ± 0.05	n.g.
Glc + Pyr	4.8 ± 0.1	0.71 ± 0.02	0.64 ± 0.01	4.6 ± 0.1	0.10 ± 0.03	n.g.

pH_i: initial pH. pH_f: final pH. n.g.: not grown.

determined in order to assess if observed growth in M17P is attributable to residual glucose present in the overnight pre-culture (Figs. 2A and 3A, filled circle versus filled triangle). Increased DOmax in M17P with respect to M17 basal medium indicates that pyruvate addition is responsible for the observed increment.

Pyruvate consumption was determined during growth of wild type and mutant strains in M17P medium. As shown in Fig. 2B, at initial pH 5.5 the IL1403 strain shows an increase in the rate of pyruvate consumption ($rate_{Pyr} = 1.9$ mM h^{-1}) in the presence of glucose, whereas the *als* mutant shows a lower rate ($rate_{Pyr} = 0.8$ mM h^{-1}, Fig. 2E). When pH was set to a value of 4.5 units (Fig. 3B), we observed a similar increase in pyruvate consumption for the wild type strain when both compounds are present in the medium ($rate_{Pyr} = 1.7$ mM h^{-1}). In contrast, the *als* mutant was not capable of consuming pyruvate under this pH condition, even in the presence of glucose in the medium (Fig. 3E). These results suggest that when both carbon sources were added to the medium at acidic pH *L. lactis* was capable of their cometabolism and that the *als* gene is required for pyruvate utilization especially at low pH.

3.2. End products of pyruvate metabolism in resting cells of *L. lactis* under acidic conditions

The metabolic end product profile of wild-type and *als*-mutant resting cells was also determined. Lactococcal cells were resuspended at OD of 1.5 in 50 mM phosphate solution adjusted to 4.5 or 5.5 pH units. Glucose was supplemented at a concentration of 5 mM and pyruvate at 20 mM. Glucose metabolism in resting cells of IL1403 and ILGR1 strains,

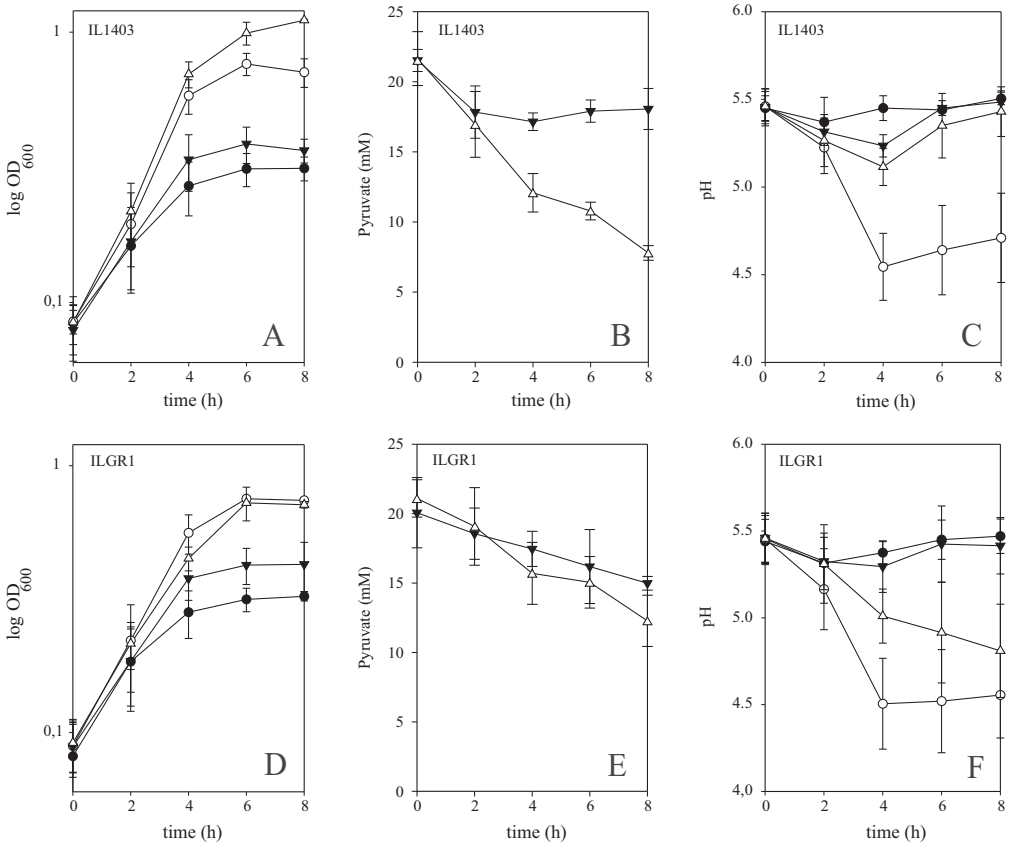

Fig. 2. Growth of *L. lactis* IL1403 and ILGR1 in M17 adjusted to pH 5.5 (A and D, respectively). pH remained uncontrolled during the assay. Strains were grown in M17 basal medium (filled circle) or the same medium supplemented with 11 mM glucose (empty circle), 25 mM pyruvate (filled triangle) or both carbon sources (empty triangle). Pyruvate consumption was also followed for both strains during the assay (B and E, respectively). External pH variations (C and F, respectively). Experiments were assayed in triplicate and data presented corresponds to the mean value ± standard deviation.

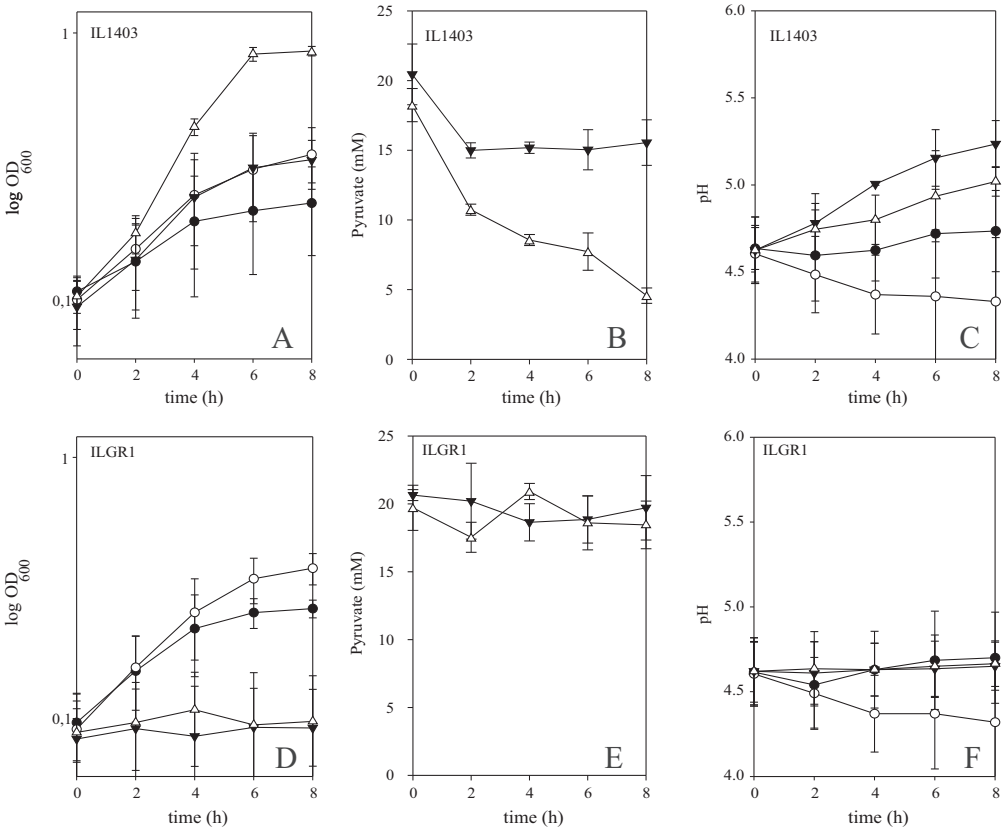

Fig. 3. Growth of *L. lactis* IL1403 and ILGR1 in M17 adjusted to pH 4.5 (A and D, respectively). pH remained uncontrolled during the assay. Strains were grown in M17 basal medium (filled circle) or the same medium supplemented with 11 mM glucose (empty circle), 25 mM pyruvate (filled triangle) or both carbon sources (empty triangle). Pyruvate consumption was also followed for both strains during the assay (B and E, respectively). External pH variations (C and F, respectively). Experiments were assayed in triplicate and data presented corresponds to the mean value ± standard deviation.

as expected, showed homofermentative metabolism, producing only lactate at both initial pH values of 4.5 or 5.5 units (Table 4).

Addition of pyruvate alone to the wild-type resting cells showed a differential product pattern with lactate and acetate as the main end products. Acetoin was detected in trace amounts while formiate, ethanol, 2,3-butanediol and diacetyl were not detected (Table 4). In contrast, pyruvate metabolism was not observed for the *als* mutant strain, neither at pH 4.5 nor at pH 5.5 (Table 4). When glucose and pyruvate were simultaneously added to resting cells of the IL1403 strain, products observed were mainly lactate followed by acetate and acetoin. Similar amounts of lactate were produced by cells at both pH values. However, the production of acetoin was maximal at pH 4.5 whereas acetate levels were higher at pH 5.5. On the other hand, resting cells of the *als*-defective strain showed a similar product pattern regarding lactate and acetate generation at both pH conditions, whereas formiate, ethanol, 2,3-butanediol acetoin and diacetyl were not detected. These experiments showed that lactococcal cells are capable of metabolizing pyruvate by means of the LDH, ALS and the PDH-PTA pathway (Fig. 1), but the presence of ALS is essential for C4-compound production in acidic conditions.

Table 4
End-product determination in resting cells[a] of *L lactis* strains.

		Concentration (mM) at 120 min[b]			
	pHi	Pyruvate	Acetate	Lactate	Acetoin
IL 1403					
Glc	5.5	n.d.	n.d.	8.4 ± 1.1	n.d.
Pyr	5.5	17.85±	1.0 ± 0.1	1.2 ± 0.0	0.1 ± 0.0
Glc + Pyr	5.5	18.8 ± 0.3	0.5 ± 0.1	7.1 ± 0.1	0.4 ± 0.1
Glc	4.5	n.d.	n.d.	5.6 ± 0.5	n.d.
Pyr	4.5	17.45 ± 0.9	1.0 ± 0.2	1.8 ± 0.2	0.25 ± 0.1
Glc + Pyr	4.5	17.6 ± 0.7	0.2 ± 0.0	7.1 ± 0.5	0.9 ± 0.1
ILGR1					
Glc	5.5	n.d.	n.d.	8.3 ± 0.3	n.d.
Pyr	5.5	19.9 ± 0.1	n.d.	n.d.	n.d.
Glc + Pyr	5.5	19.7 ± 0.1	0.5 ± 0.0	6.1 ± 0.3	n.d.
Glc	4.5	n.d.	n.d.	7.4 ± 1.1	n.d.
Pyr	4.5	20.0 ± 0.2	n.d.	n.d.	n.d.
Glc + Pyr	4.5	19.6 ± 0.2	0.6 ± 0.1	4.9 ± 0.6	n.d.

[a] Concentrations are given in mM. Cells were grown overnight in M17G pH 5.5 and then resuspended at OD of 1.5 in 50 mM phosphate solution adjusted to 5.5 or 4.5 pH units supplemented with the indicated carbon source (5 mM glucose or 20 mM pyruvate). n.d.: not detected.

[b] = Formiate, ethanol, 2,3-butanediol and diacetyl were not detected.

3.3. Comparison of external and internal pH levels between wild type *L. lactis* and als mutant strains

To determine if the observed growth impairment of the *als* mutant was related to its inability to counteract acidification, we decided to evaluate changes in external pH in batch cultures of IL1403 and ILGR1 cells (Figs. 2 and 3, and Table 3). As shown in Fig. 2C and F, when grown at pH 5.5 glucose metabolism (empty circle) provoked a maximum acidification of the external medium up to values near 4.5 units, for both strains. Conversely, when only pyruvate (filled triangle) was added as carbon source, none of the strains caused a significant acidification of the growth medium and the final pH value remained approximately the same as the initial, after diminishing 0.2 units at the onset of growth. A similar behavior was observed for the wild type strain in M17GP (empty triangle), with an acidification of external pH until reaching 5.1 units after 4 h, returning to a value of 5.5 at the end of

the culture period. On the other hand, after showing a similar initial acidification, the external pH of the ILGR1 strain culture continued decreasing until a value of 4.8 units after 8 h (Fig. 2 F), i.e. for the *als* deficient strain the external pH decreased 0.7 units more than that of the wild type strain. Accordingly, the *als* mutant reached a lower final OD than the wild type (Table 3). When the initial pH was set to 4.5, the strain IL1403 showed an acidification of 0.3 units in M17G (empty circle), whereas an increase of 0.6 units was seen when pyruvate (filled triangle) was added to the medium (Fig. 3C). In the presence of both compounds (empty triangle), an equilibrium between both tendencies was observed, which led to a final pH value of 4.8 units (Fig. 3C). In contrast, even when the strain ILGR1 showed a similar acidification behavior compared to the wild type in M17G, it was unable to alkalinize the medium when grown in the presence of pyruvate, regardless of glucose addition (Fig. 3F). Therefore, the *als*-deficient strain incapacity for pyruvate assimilation under these conditions led to the observed growth impairment.

In order to study the contribution of the ALS enzyme to pH homeostasis in acidic medium, internal H^+ levels were monitored by using the pH-sensitive fluorescent probe BCECF. *L. lactis* wild type and *als* mutant cells were loaded with the fluorescent probe, suspended in phosphate buffer (pH 4.5) and a pulse of the indicated carbon source was subsequently added to the suspension. Resting cells maintained an internal pH between 5.3 and 5.4 when suspended in this buffer. First, IL1403 cells were subjected to a pyruvate pulse. This led to a cytoplasmic acidification, with a subsequent alkalinization up to a final internal pH of 5.5, after 2 min (Fig. 4, dashed black line). Then, the glucose addition 2 min later produced an additional alkalinization of cytoplasmic pH. On the other hand, internal and external pH values remained unaltered (4.5) when the *als* mutant was subjected to a pyruvate pulse (Fig. 4, solid black line). These levels of internal pH acidification could not be counteracted even when cells were subjected to a glucose pulse (indicated by Glu arrow). To confirm arrest of the metabolic route in the *als*-defective strain, the internal pH was raised after the pyruvate pulse by direct addition of 0.1 N NaOH into the cuvette to achieve the initial pH (indicated by asteriks). As shown in Fig. 4 (gray solid line), this strain becomes responsive to glucose addition after the artificial pH increase. These results underpin the importance of internal alkalinization undertaken by pyruvate utilization through the C4 pathway, in order to favor glucose consumption under these conditions.

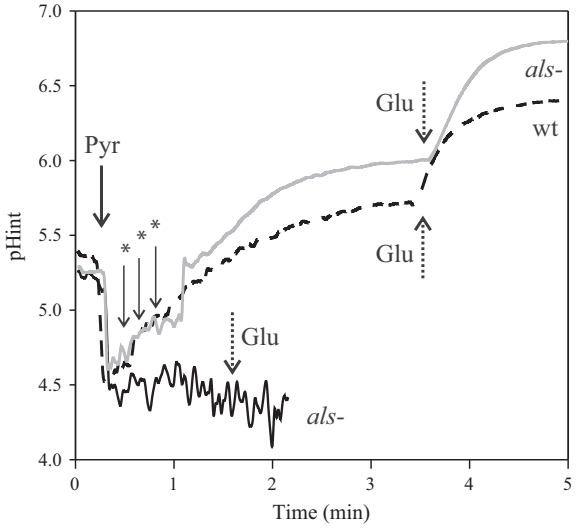

Fig. 4. *L. lactis* internal pH variations in response to pyruvate. IL1403 (dotted line, wt) and ILGR1 (continuous line, *als*⁻) strains were grown in M17G up to exponential phase. Then, cells were loaded with the BCECF fluorescent probe and resuspended in phosphate buffer at 4.5. Pulses of 50 mM pyruvate (pyr), 3 mM glucose (glu) or 0.1 N NaOH (*) were added at the times indicated by the arrows (gray line).

All these experiments suggest that by redirecting the accumulated pyruvate to the C4 route the wild type strain achieves an increase in the internal pH, which makes an important contribution to pH homeostasis. In turn, this leads to a biomass increment when this compound is cometabolized with sugars at the acidic pH values in which LAB perform fermentation.

3.4. Genomic organization of the C4 pathway in *L. lactis*

Taking into account the importance of aroma-compound production for the dairy industry, a bioinformatic analysis was conducted in order to get more information about the evolutionary process of genes involved in citrate and pyruvate catabolism, and in diacetyl/acetoin production. By using a comparative genomic approach we found that the presence of the *als* gene is conserved in all strains of *L. lactis* analyzed (indicated by * in Fig. 5). The gene context analysis revealed that the putative *yljGHIJ* operon is conserved in all strains of *L. lactis* upstream of the *als* gene. Interestingly, the main difference occurs for genes downstream of *als*, which present a high variability. Among the *lactis* subspecies and more specifically the IL1403 strain (derived from a diacetylactis strain), there has been an insertion of approximately 23 kbp downstream of *als* that includes the complete set of genes required for citrate fermentation. This cluster harbors genes for a soluble oxaloacetate decarboxylating enzyme CitM, the transcriptional factor CitI, the citrate lyase complex and a pseudogene encoding a putative citrate transporter (Martin et al., 2004, 2005). In the case of the strain KF147, this insertion corresponds to a fragment of about 60 kbp and includes the *pks* operon involved in the synthesis of polyketide compounds. In the human isolate CV56, a 6 kbp insertion is present (with genes not clearly associated to any particular phenotype), while for the industrial OI-1 strain no insertion was detected in this region (Fig. 5). Despite these insertions detected for some of the *L. lactis* subsp *lactis* strains, no other reorganizations regarding other genes involved in pyruvate metabolism or the C4 compound biosynthetic route were observed (Fig. 5). For strains belonging to the *cremoris* subspecies, the main difference is not related to the insertion of a DNA fragment adjacent to the *als* gene but to the genetic diversity among them, which is due to an intense reorganization of the genes involved in the C4 pathway (Fig. 5).

In conclusion, the genes required for the synthesis of C4 compounds are conserved in *L lactis*. However, there is a high variability in the genomic organization among the microorganisms studied.

4. Discussion

L. lactis is one of the major worldwide starter cultures used in artisanal and commercial dairy industry. During milk processing, lactococcal cells are able to survive under acidic stress. This, and other traits (such as aroma production), are the reason why many strains were selected. In this paper, genetic evidence is presented for the first time to demonstrate that *als* is directly involved in *L. lactis* pH homeostasis. The contribution of the acetoin/diacetyl pathway is evident at pH 4.5, which constitutes a common acidic stress situation for *L. lactis* growth. The experiments revealed that during the co-metabolism of sugars and pyruvate under acidic conditions the generation of aroma compounds plays an important role for bacterial growth. Under these conditions, the wild-type IL1403 strain was able to grow and cometabolize pyruvate and glucose whereas the *als* mutant (ILGR1) was not. In the presence of pyruvate, an active alkalinization of external pH is observed for the wild-type strain, which correlates well with the analysis of internal pH in real time using a fluorescent probe (Magni et al., 1999; Espariz et al., 2011). To date, no pyruvate transporter was described in LAB and it is proposed that the protonated form of pyruvate (pyruvic acid, 2.4 pKa) presumably diffuses through the membrane at low pH. Under acidic conditions, organic acids act as inhibitory compounds of microorganism growth due to a protonophore effect (Cotter and Hill, 2003). Clearly, we present evidence favoring pyruvate

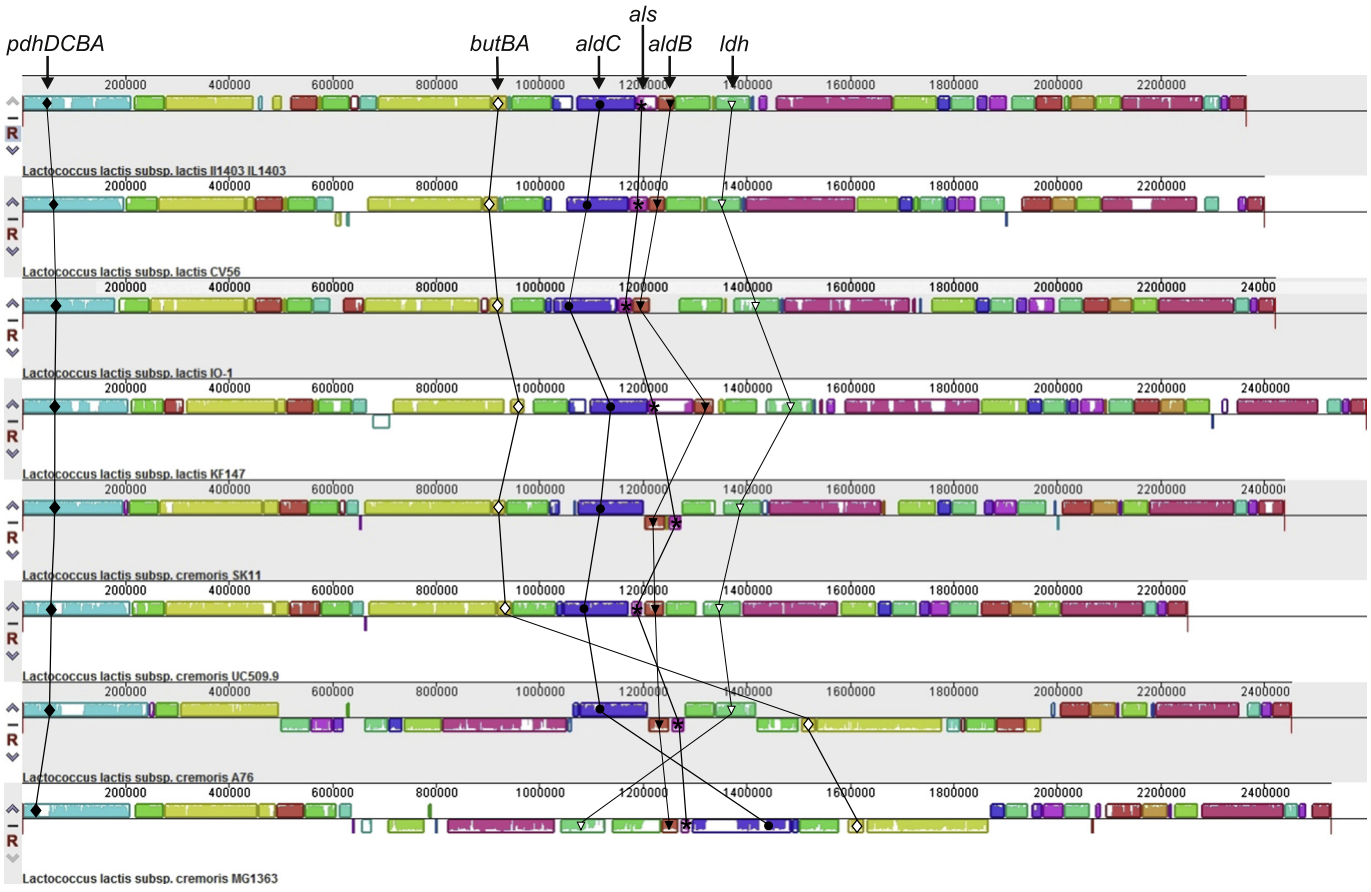

Fig. 5. Genetic organization of pyruvate metabolism genes in different subspecies of *L. lactis*.

degradation in *L. lactis*, which produces an increase in the level of C4 compounds synthesized but also results in a contribution of the pathway to the alkalinization of the internal and external pH by scalar proton consumption (Fig. 1; Lolkema et al., 1995). Also in this work, we analyzed the effect of *als* mutation in resting cell experiments. *L. lactis* showed a homofermentative metabolism in the presence of glucose in both wild type and mutant strains, and therefore only lactate was detected (Table 4). Addition of pyruvate to wild-type resting cells produced lactate, acetate and acetoin while, as expected, no C4 compound was detected for the *als* mutant. We hypothesize that acetate detected under these conditions could be produced by the action of the PDH complex (Table 4). No formiate or ethanol was detected, which strongly suggests that under our experimental conditions alternative pathways for pyruvate metabolism are not operative. Similar results were obtained for resting cells of the IL1403 strain during the cometabolism of glucose and pyruvate at low pH. The wild type produced lactate, acetate and acetoin while the mutant is unable to produce the latter compound.

Given the importance of the mentioned pathways for the dairy industry, we additionally performed a genome-scale analysis on different isolates of *L. lactis*. Our data shows that the C4 pathway was present in *L. lactis* species previous to the differentiation into the *lactis* and *cremoris* subspecies. While in the *lactis* subspecies, it is possible to find the insertion of different DNA segments downstream of the *als* gene, in the *cremoris* subspecies, inversions and internal reorganization of pathway genes are observed. These results support the designation for this strain as biovar. citrate instead of biovar. diacetylactis, as previously proposed Passerini et al. (2013).

Based on the fact that *L. lactis* IL1403 is a diacetylactis-derived strain (Table 1, Górecki et al., 2011) and has conserved the genetic context extensively characterized for CRL264, the model diacetylactis strain, we speculate that two different genetic events occurred during the evolutionary process for the acquisition of the citrate fermentation cluster (relevant trait of the biovar.). First, insertion of the *cit* cluster adjacent to *als* is observed. As previously described, the *citM-citO-citI-CitCDEFXG* cluster carries the genes encoding the citrate lyase, the soluble OAD, the transcriptional activator CitI and also includes a pseudogene encoding a putative citrate trasporter (*citO*) which accumulates a high number of mutations (Martin et al., 2004). The *cit* operon is induced at low pH and the corresponding gene products constitute a citrate degradation pathway, yielding pyruvate. This genetic event could have been provoked through recombination mediated by a composite transposon; it is possible to find full and partial insertion sequences flanking the insertion in the genome of IL1403 or CRL264 strains (Bolotin et al., 2001; Martin et al., 2004). Secondly, this strain could have recently acquired the plasmidic gene encoding a citrate transporter CitP (8.5 kbp plasmid: pCit264 in the CRL264 strain or pIL2 in the IL594 strain) (Górecki et al., 2011; López de Felipe et al., 1996; Sesma et al., 1990). It is tempting to speculate that pIL2 and pCIT264 (and other pCit plasmids carrying the *citP* gene) could have been originated by a deletion of the catabolic cluster *citI-citMCDEFGRP* from the larger 23 kbp plasmid (as is the case with pCitJ from *Weissella paramesenteroides*) (Martin et al., 1999, 2000). Evidence favoring this hypothesis is given by the fact that plasmid-encoded *citP* genes share 99% of identity. The presence of both types of plasmids was recently described in lactococcal strains isolated from dromedaries, nevertheless no full genomic nucleotide sequence data was available for these strains to be included in our comparative analysis (Drici et al., 2010). Altogether, these results prove that the C4 pathway is not directly associated to citrate metabolism although its regulation is induced at low pH (García-Quintans et al., 1998, 2008). In this microorganism, citrate and pyruvate metabolism via *als* constitute an acidic stress response. This capacity is conserved in all strains studied, whereas the ability to metabolize citrate is conserved in a small number of *L. lactis*

subspecies *lactis* (which should be described as biovar. citrate and not as biovar. diacetylactis). In conclusion, we have demonstrated that pyruvate metabolism at low pH requires a functional ALS, since this pathway is interconnected with pH homeostasis. Pyruvate transformation into α-acetolactate, and its subsequent decarboxylation, produces an increase in intracellular pH for the wild type strain which allows the activity of i) glycolytic enzymes and thus ATP generation by means of glucose co-metabolism and ii) the function of the membrane-bound F-ATPase (H^+-translocating ATPase) which is considered the primary determinant of acid tolerance in *L. lactis* (Cotter and Hill, 2003). In this sense, pyruvate co-metabolism via ALS restores intracellular pH homeostasis and drives the growth of wild-type lactococcal cells whereas for the *als*-deficient strain glycolysis and ATPase activity are completely inhibited leading to cell growth arrest.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.017.

Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT2010-1828 and PICT 2008-1562, Argentina), CONICET (PIP 2012-2014) and a European Union grant (BIAMFood, contract KBBE-211441). FAZ is a fellow of ANPyCT, GDR is a fellow of CONICET; SHA and CM are researchers of the same institution.

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