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ORIGINAL PAPER

# Analysis of *ldh* genes in *Lactobacillus casei* BL23: role on lactic acid production

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**Abstract** *Lactobacillus casei* is a lactic acid bacterium that produces L-lactate as the main product of sugar fermentation via L-lactate dehydrogenase (Ldh1) activity. In addition, small amounts of the D-lactate isomer are produced by the activity of a D-hydroxycaproate dehydrogenase (HicD). Ldh1 is the main L-lactate producing enzyme, but mutation of its gene does not eliminate L-lactate synthesis. A survey of the *L. casei* BL23 draft genome sequence revealed the presence of three additional genes encoding Ldh paralogs. In order to study the contribution of these genes to the global lactate production in this organism, individual, as well as double mutants (*ldh1 ldh2*, *ldh1 ldh3*, *ldh1 ldh4* and *ldh1 hicD*) were constructed and lactic acid production was assessed in culture supernatants. *ldh2*, *ldh3* and *ldh4* genes play a minor role in lactate production, as their single mutation or a mutation in combination with an *ldh1* deletion had a low impact on L-lactate synthesis. A *Δldh1* mutant displayed an increased production of D-lactate, which was probably synthesized via the activity of HicD, as it was abolished in a *Δldh1 hicD* double mutant. Contrarily to HicD, no Ldh1, Ldh2, Ldh3 or Ldh4 activities could be detected by zymogram assays. In addition, these assays revealed the presence of extra bands exhibiting D-/L-lactate dehydrogenase activity, which could not be attributed to any of the described genes. These results suggest that *L. casei* BL23 possesses a complex enzymatic system able to reduce pyruvic to lactic acid.

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## Introduction

The metabolism of sugars by lactic acid bacteria (LAB) is characterized by the production of lactate as the main fermentation product via the action of lactate dehydrogenase, which reduces pyruvic acid to lactic acid. Lactic acid production is important from a biotechnological point of view, as it can be produced by LAB fermentation of many natural sources and it can be used in the food, pharmaceutical and biopolymers industries [7]. In *Lactobacillus casei* BL23, a strain that has been widely used for genetic, physiological and biochemical studies, two genes encoding proteins with lactate dehydrogenase activity have been described [8, 12, 19]. Gene *ldh1* codes for an L-Ldh responsible for the synthesis of L-lactate, whilst *hicD* encodes a D-hydroxyisocaproate dehydrogenase that provides D-lactate. Mutant strains have been constructed in both genes demonstrating that they were responsible for the main L- and D-lactate formation in this bacterium [19]. However, an *L. casei* BL23 *ldh1* mutant still produced substantial amounts of L-lactate and the production of D-lactate was increased. A comparable behaviour has also been reported for other LAB with deleted *ldh* genes. In this sense, mutation of the genes encoding L- and D-Ldhs from *Lactobacillus plantarum*, an organism which produces a mixture of 50% D- and 50% L-lactate, never resulted in a complete lack of lactate production [3]. An *ldhL* mutation in *Lactobacillus sakei*, a lactic acid bacterium which lacks D-lactate dehydrogenase activity, resulted in a strain with strongly reduced L- and D- lactate production (the D isomer was a consequence of the

presence of a racemase activity able to transform L- into D-lactate) but small amounts of lactate were still produced [15]. Finally, a *Lactobacillus fermentum ldhL-ldhD* double knockout was still able to synthesize lactate [1]. Efforts have been made to construct LAB strains affected in one or several of the identified *ldh* genes, as they can be used in the production through fermentation of non-racemic, optically active lactic acid [1]. In addition, metabolic engineering strategies were applied where oxidation of NADH was coupled to an alternative metabolism of pyruvate, leading to the production of value-added metabolites [4, 6, 13, 17]. However, deletion of known lactate dehydrogenase genes in combination with the expression of enzymatic activities able to reduce pyruvate is not always sufficient to divert a significant pyruvate flow towards other molecules of interest different from lactic acid. For those reasons, a better study of the enzymes implicated in lactate synthesis becomes necessary. In an attempt to discover the genes responsible for residual lactic acid production in an *L. casei* BL23 *ldh1* mutant, we searched its genome for the presence of additional *ldh* genes and studied the effects of mutations in the newly found *ldh*s on lactate production.

## Material and methods

### Strains and growth conditions

The *L. casei* strains used in this study are listed in Table 1. They were grown in MRS medium (Oxoid) or MRS basal medium, containing per litre: peptone, 10 g; meat extract, 8 g; yeast extract, 4 g; Tween 80, 1 mL; $K_2HPO_4$, 2 g; ammonium citrate, 2 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.4H_2O$, 0.05 g and supplemented with 0.2% (w/v) glucose, at 37 °C under static conditions. *E. coli* DH5α was used as a cloning host and it was grown in LB medium at 37 °C under agitation. For selecting *L. casei* transformants, erythromycin was added to the growth medium at 5 μg/mL. Ampicillin was used at 100 μg/mL for *E. coli*.

### Construction of plasmids and mutant strains

The pRV300 plasmid [11] was used to construct integrative vectors carrying internal fragments of *L. casei ldh2*, *ldh3* and *ldh4*. A 519 bp fragment from *ldh2* was amplified by PCR with oligonucleotides 5′-GATTCCCTACCTTA CACG and 5′-TCTCAATGATGCCATAAGC and cloned into pRV300 digested with EcoRV, giving pRVldh2. A 505-bp *ldh3* internal fragment was amplified with 5′-GTG TTATTTCCGCGGTC and 5′-GCTTAGCCTGATAAA TCTTC and cloned into pRV300 digested with SmaI to give pRVldh3. A 437-bp fragment of *ldh4* was amplified with 5′-TCTCAATTCAGCGATGGC and 5′-CTACATC ATCAGATGCG and cloned into EcoRV-digested pRV300 to give pRVldh4. To construct the pRVΔldh1 plasmid carrying fused 5′ and 3′ *ldh1* regions, a 500-bp fragment corresponding to the *ldh1* 3′ downstream region was amplified with oligonucleotides 5′-CGACACCGAGA ATTTCTGTG and 5′-ACATGGATGGTCAATATGGC and cloned into pRV300 digested with EcoRI (made blunt with Klenow fragment of DNApol I). Another 500 bp from the 5′ upstream region of *ldh1* was amplified with oligonucleotides 5′-CGGGGTACCGTCGTTTGGCCAAGCCATC and 5′-TTCAAGCAAGCTTCCAATAAC and cloned into the above plasmid digested with PstI (made blunt with Klenow). A clone containing the resulting plasmid with the fragments in the proper orientation was identified by restriction analysis and named pRVΔldh1. PCRs were carried out with Expand High Fidelity polymerase (Roche) and chromosomal DNA from *L. casei* BL23. PCR products were gel-purified with the GFX-PCR purification kit (GE Healthcare). Restriction enzymes, DNA modification enzymes and T4 ligase were from New England Biolabs and Roche.

*Lactobacillus casei* strains were transformed with pRVldh2, pRVldh3, pRVldh4 and pVBhic [19] by electroporation with a GenePulser apparatus (BioRad) and transformants were selected on MRS plates containing erythromycin. Integration at the correct locus was tested by PCR with appropriate oligonucleotides and DNA of the transformants. To construct an *ldh1* deletion strain, *L. casei* BL23 was transformed with pRVΔldh1 and erythromycin-resistant clones were selected. One of these clones was grown for about 200 generation without antibiotic and dilutions were plated on MRS and replica-plated on MRS containing erythromycin. BL249 (*Δldh1*) was selected as a strain in which a second recombination event led to the excision of the plasmid leaving a deletion of *ldh1*, as was confirmed by PCR performed with DNA isolated from erythromycin-sensitive clones.

### Measurement of organic acids and glucose

*Lactobacillus casei* cells were cultured to early stationary phase in MRS basal medium supplemented with 0.2% glucose. The organic acids were measured in the supernatants with a Jasco PU-2080Plus HPLC system coupled to an UV detector (210 nm) and using a Rezex ROA-Organic Acid column. Chromatography was carried out at 50 °C with 5 mM $H_2SO_4$ as a solvent and at a flow rate of 0.6 mL/min. The proportion of D- and L-lactate isomers was determined with the D-/L-lactic acid kit from R-Biopharm (Darmstadt, Germany). Glucose present in the growth medium was measured with the D-Glucose kit from R-Biopharm.

**Table 1** Strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source or reference |
|---|---|---|
| *Lactobacillus casei* | | |
| BL23 | Wild-type, genome sequenced at the Université de Caen, CNRS[a], INRA[b] and CSIC[c] | B. Chassy, U. Illinois |
| BL176 | BL23 *ldh1*::pRV300 | [19] |
| BL198 | BL23 *hicD*::pRV300 | [19] |
| BL249 | BL23 *Δldh1* | This work |
| BL252 | BL23 *Δldh1 ldh2*::pRV300 | This work |
| BL269 | BL23 *ldh2*::pRV300 | This work |
| BL270 | BL23 *ldh3*::pRV300 | This work |
| BL271 | BL23 *Δldh1 ldh3*::pRV300 | This work |
| BL272 | BL23 *ldh4*::pRV300 | This work |
| BL273 | BL23 *Δldh1 ldh4*::pRV300 | This work |
| BL274 | BL23 *Δldh1 hicD*::pRV300 | This work |
| Plasmids | | |
| pRV300 | Insertional vector for *Lactobacillus*, Amp$^r$, Ery$^r$ | [11] |
| pRVΔldh1 | pRV300 with a 1 kb DNA fragment carrying fused 5′ and 3′ *ldh1* regions | This work |
| pRVldh2 | pRV300 with a 0.5 kb *ldh2* fragment cloned at EcoRV site | This work |
| pRVldh3 | pRV300 with a 0.5 kb *ldh3* fragment cloned at SmaI site | This work |
| pRVldh4 | pRV300 with a 0.4 kb *ldh4* fragment cloned at EcoRV site | This work |
| pVBhic | pRV300 with a 0.6 kb *hicD* fragment cloned at SmaI site | [19] |

[a] Centre National de la Recherche Scientifique

[b] Institut National de la Recherche Agronomique

[c] Consejo Superior de Investigaciones Científicas

### Lactate dehydrogenase zymogram assays

*Lactobacillus casei* wild-type and mutant strains were grown in 10 mL of MRS and exponentially growing cells were recovered by centrifugation, washed with Tris–HCl 100 mM pH 7.4 and resuspended in the same buffer supplemented with 1 mM dithio-1,4-threitol and 0.5 mM phenylmethylsulphonyl fluoride. Cells were broken by shaking with glass beads (0.1 mm diameter) in a Mini-Beadbeater (Biospec) and cellular debris was removed by centrifugation 10 min at 12,000×*g* and 4 °C. Cellular extracts were loaded onto a 6% non-denaturing PAG and resolved at 90 V. To detect lactate dehydrogenase activity the gels were incubated in 100 mM triethanolamine buffer pH 6.8 containing 0.75 mM $NAD^+$, 0.1 mg/mL nitroblue tetrazolium, 0.02 mg/mL phenazyne methosulfate and either 200 mM L-lactate or 200 mM of a 50% D-50% L-lactate mixture. In a second assay electrophoresis was carried out with a 10% PAG containing 0.05% SDS. The gels were soaked for 60 min in 2% Triton X-100 to eliminate the SDS and transferred to a solution containing 100 mM triethanolamine buffer pH 6.8 with 10 mM fructose-1,6-bisphosphate, 1 mM NADH with or without 25 mM sodium pyruvate. After 60 min of incubation, the gels were rinsed with water for 30 min and incubated in a solution containing 0.25 mg/mL nitroblue tetrazolium and 0.02 mg/mL phenazyne methosulfate. Protein bands with NADH oxidase activity were evidenced as clear bands over a dark background.

### Nucleotide accession numbers

The nucleotide sequences reported in this paper have been deposited at the EMBL database under accession numbers AM886174, AM886175, AM886176 and AM886177.

## Results

### *ldh* homologues present in the *L. casei* BL23 genome

In addition to the previously described Ldh from *L. casei* BL23 [8, 19], a BLAST search for *ldh* homologues in the *L. casei* BL23 draft genome (96% sequence coverage) rendered three additional genes encoding putative Ldhs. The three additional Ldhs (Ldh2, Ldh3 and Ldh4) had a percentage of identity of 49, 31 and 24%, respectively, when compared to the *L. casei* BL23 Ldh1 enzyme. Ldh2 had homologies to lactate/malate dehydrogenase enzymes, whereas Ldh3 was most similar to L-hydroxyisocaproate dehydrogenases from many bacteria. In Ldh4, sequence homology to other L-Ldhs started at around aminoacid 80, whereas the first N-terminal amino acids only shared a significant homology to the N-terminus of the secondary Ldh from *L. lactis* (Ldh-2). Sequence analysis of the putative Ldhs revealed that, similar to Ldh1, the Ldh2 and Ldh3 proteins carried conserved NADH-binding domains containing the typical G-X-G-X-X-G pattern (X is any amino

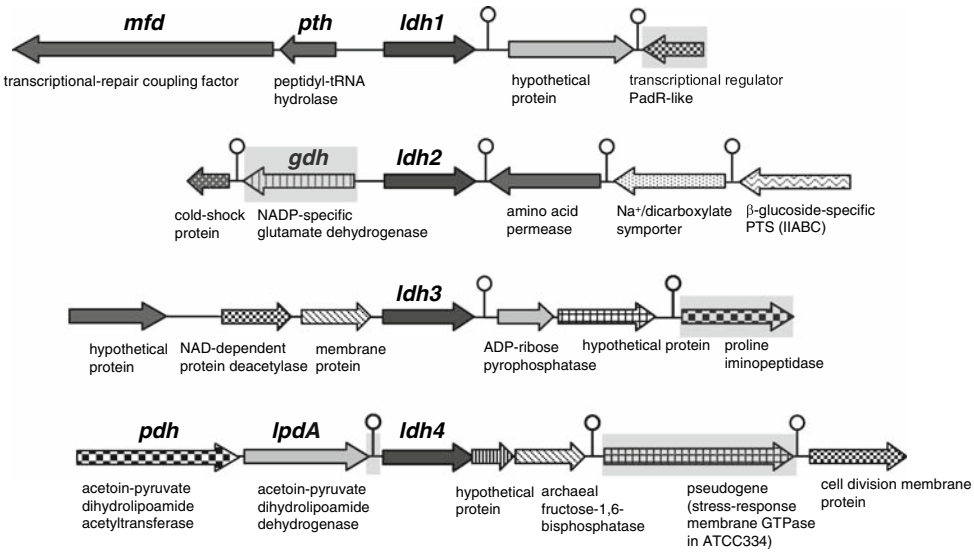


**Fig. 1** Schematic representation of the four different *ldh* genes detected in *L. casei* BL23 together with their genome context. *Grey boxes* correspond to sequences which differ from *L. casei* ATCC334. Stem-loops represent putative transcriptional terminators

acid). Furthermore, the catalytic M-G-E-H-G-[D/E]-[S/T] site, where H is the catalytic histidine, was also present in Ldh2 and Ldh3, supporting thus the hypothesis that they encode potential Ldh enzymes. Ldh4 was the enzyme exhibiting the lowest similarity to Ldh1 and conservation of the above sequence motifs was poor. In order to assign putative roles to the new proteins, the genetic contexts of their corresponding genes were studied (Fig. 1). As previously described, *ldh1* is a monocistronic gene and no genes related to carbon metabolism are located next to it. *ldh2* is flanked at its 5′-end by the divergently transcribed *gdh* gene, encoding a NADP-dependent glutamate dehydrogenase and at its 3′ end by genes encoding putative amino acid and dicarboxylic acid transporters. Although it appears that *ldh2* is also a monocistronic gene, it is plausible that *ldh2* carries a function in the dehydrogenation step of some dicarboxylic acids. *ldh3* is flanked by genes encoding a putative deacetylase, a membrane protein and an ADP-ribose pyrophosphatase. *ldh4* is located at the 3′-end of a gene cluster encoding the different subunits of the pyruvate dehydrogenase complex and seems to form an operon with a gene encoding a conserved hypothetical protein and a putative archaeal-type fructose-1,6-bisphosphatase of the inositolmonophosphatase family. While the pyruvate dehydrogenase complex is involved in pyruvate metabolism as it converts this compound into acetyl-CoA under aerobic conditions, fructose-1,6-bisphosphatase takes part in the gluconeogenic pathway and therefore no clear link with Ldh could be recognized. In conclusion, genome context of the new *ldh* genes did not allow establishing likely hypotheses about their cellular roles. A similar search carried out by using the complete genome sequence of *L. casei* ATCC334 [14], the newly proposed *L. casei* type-strain, revealed the occurrence of the same additional *ldh* genes. The genetic structure of the DNA regions carrying the *ldhs* was identical in both strains except that: (1) in ATCC334, the *gdh* gene (LSEI_0635) adjacent to *ldh2* and the putative proline iminopeptidase gene (LSEI_2604) close to *ldh3* are pseudogenes; (2) an IS3-related insertion element is present in ATCC 334 between *lpdA* and *ldh4*; (3) the gene encoding a putative PadR-like regulator in BL23 is absent from ATCC 334; and (4) the gene encoding a stress-response membrane GTPase in ATCC334 (LSEI_1313) is a pseudogene in BL23 (Fig. 1).

## Construction of different *ldh* mutants and their effect on lactate production

In a previous work, an *ldh1* mutant was constructed by insertion of a non-replicative plasmid carrying an internal fragment of the gene [19]. In order to produce a stable mutant and to avoid the presence of an antibiotic resistance marker, *L. casei* BL23 was transformed with plasmid pRVΔldh1, which carried the 5′ and 3′ flanking regions of *ldh1*. After a first single-crossover integration, a stable mutant was selected which underwent a second recombination event, thus leading to a complete deletion of *ldh1* in the chromosome. When compared to the wild-type, the new deletion mutant (strain BL249) exhibited a behaviour similar to that of the previous insertional mutant BL176: culture supernatants always reached a higher final pH, it produced $CO_2$ from glucose and L-Ldh activity in crude extracts was reduced by 95% (data not shown). Subsequently, the *ldh2*, *ldh3*, *ldh4* and *hicD* genes were inactivated in the *Δldh1* background giving four double mutants (Table 1). In a similar way, the *ldh2*, *ldh3* and *ldh4* genes were inactivated in the *L. casei* wild-type, giving three different single mutants (Table 1).

In order to study the effect of the different mutations on lactic acid production, all strains were grown in MRS basal medium containing 0.2% glucose and lactic acid

concentrations were measured after complete glucose utilization. As can be seen in Table 2, a *Δldh1* mutation had a major impact on lactate production, with a reduction of 37% in total lactic acid, whereas an increase in the percentage of D-lactate was observed. The *ldh2*, *ldh3* and *ldh4* single mutants behaved like the wild-type (data not shown). Furthermore, combination of *ldh2*, *ldh3* or *ldh4* mutations with an *ldh1* deletion did not produce a clear effect on total lactate production, although the D-/L-lactate ratio increased more than twofold in the *ldh1 ldh2* and *ldh1 ldh4* double knockouts compared to the single *ldh1* mutant. These results suggested that in the absence of Ldh1 and either Ldh2 or Ldh4, HicD or other D-lactate producing enzymes might reduce pyruvate at a higher rate (Table 2). Interestingly, the increased D-lactate production in the *Δldh1* strain completely disappeared in a *Δldh1 hicD* double mutant. This confirmed that, in the absence of Ldh1, the HicD enzyme was responsible for channelling part of the pyruvate towards the production of D-lactate. As previously observed, the *ldh1* mutation caused an increase in the production of pyruvate and acetate during glucose fermentation [19]. However, in the *hicD* mutant, the small production of D-lactate of the wild-type was still observed.

## Lactate dehydrogenase activities detected in zymograms

The low effect of *ldh2*, *ldh3* and *ldh4* mutations on lactate synthesis prompted us to look for the presence of additional Ldh activities present in *L. casei* BL23 which we tried to detect by zymogram analyses of crude extracts. In a first series of experiments, cellular extracts of several strains were loaded onto non-denaturing gels and the ability to oxidize lactate was coupled to the reduction of the chromogenic substrate nitroblue tetrazolium (Fig. 2). A band exhibiting D-lactate dehydrogenase activity and which was absent in a *hicD* mutant was unequivocally detected (Fig. 2a), demonstrating that it was the product of the *hicD* gene. By the contrary, the rest of mutations did not produce a different band profile and, therefore, no protein band could be assigned to the products of either *ldh1*, *ldh2*, *ldh3* or *ldh4*. Strains carrying the single *ldh2*, *ldh3* or *ldh4* mutations behaved like the wild-type (data not shown). The use of a D-/L-lactate mixture allowed the detection of a faint

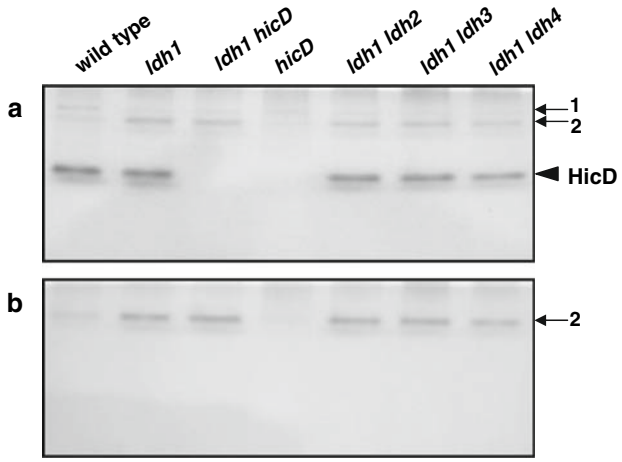


**Fig. 2** Ldh zymogram assay of different *L. casei* strains on native gels. **a** Gel developed with a 50:50 mixture of D- and L-lactate; **b** gel developed with L-lactate. The *arrows* point to protein bands exhibiting Ldh activity. The band corresponding to the HicD enzyme is marked in *panel A*. Strains are BL23 (wild-type); BL249 (*Δldh1*); BL274 (*Δldh1 hicD*); BL189 (*hicD*); BL252 (*Δldh1 ldh2*); BL271 (*Δldh1 ldh3*) and BL273 (*Δldh1 ldh4*)

band which was absent when the gel was incubated with L-lactate and that was assigned to an additional enzyme exhibiting D-lactate dehydrogenase activity (arrow 1 in Fig. 2a). In addition, a band indicating L-lactate dehydrogenase activity was detected in all gel lanes. This band did not disappear in any of the *ldh* mutants and its signal was increased in strains carrying the *Δldh1* deletion, suggesting that it corresponds to an enzymatic activity, which is induced upon *ldh1* mutation (arrow 2 in Figs. 2a, b). Similar results were obtained in the presence or absence of fructose-1,6-bisphosphate, which is an activator for Ldh1 (data not shown).

In a second set of experiments, cellular extracts were resolved by SDS-PAGE and after SDS removal, proteins in the gel were visualized by their capacity to oxidize NADH. Figure 3 shows that, again, protein bands exhibiting NADH oxidase activity in the presence of pyruvate could only be assigned to the product of *hicD*. A control gel without pyruvate gave the same results except that the HicD band was absent (data not shown). In addition, two bands exhibiting NADH oxidase activity in the absence of pyruvate were

**Table 2** Organic acids produced from glucose by several *L. casei* strains

| Strain | Wild-type | *Δldh1* | *hicD* | *Δldh1 ldh2* | *Δldh1 ldh3* | *Δldh1 ldh4* | *Δldh1 hicD* |
|---|---|---|---|---|---|---|---|
| Lactate[a] | 21.6 ± 0.2 (3–97)[b] | 13.6 ± 0.2 (35–65) | 22.9 ± 3.4 (4–96) | 18.8 ± 1.8 (54–46) | 16.6 ± 0.71 (40–60) | 14.7 ± 1.3 (54–46) | 12.1 ± 1.6 (2–98) |
| Pyruvate[a] | 0.07 ± 0.04 | 2.09 ± 0.06 | 0.14 ± 0.01 | 2.7 ± 0.05 | 3.02 ± 0.04 | 2.35 ± 0.01 | 2.67 ± 0.40 |
| Acetate[a] | 0.25 ± 0.04 | 2.09 ± 0.07 | 0.36 ± 0.09 | 2.12 ± 0.12 | 2.22 ± 0.13 | 2.29 ± 0.24 | 1.97 ± 0.23 |

[a] Concentrations are in mM

[b] Numbers in parenthesis are percentages of D- and L-lactate, respectively

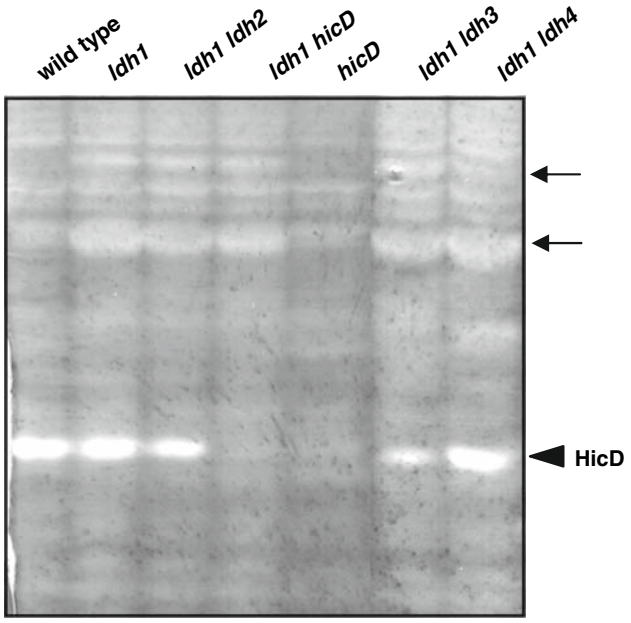


**Fig. 3** NADH oxidase zymogram assay in the presence of pyruvate. Cell-free extracts of different *L. casei* strains were resolved on an SDS-PAG which was processed as described in "Material and methods". *Arrows* point to proteins exhibiting NADH oxidase activity which was increased in a *Δldh1* background. The position of the HicD enzyme is marked with an *arrowhead*. Strains are BL23 (wild-type); BL249 (*Δldh1*); BL274 (*Δldh1 hicD*); BL189 (*hicD*); BL252 (*Δldh1 ldh2*); BL271 (*Δldh1 ldh3*) and BL273 (*Δldh1 ldh4*)

detected and their intensities were increased in a *Δldh1* background (Fig. 3).

## Discussion

Genetic modification of LAB has been used for re-routing the metabolism towards the production of industrial or food-related molecules. Mutation of Ldh-encoding genes is an important tool to achieve this goal, as it produces a redox imbalance. This can be compensated by the expression of heterologous enzymes which couples NADH oxidation to the synthesis of new compounds [4, 9, 17, 20] or which provides alternative pathways for pyruvate metabolism [6, 13, 18]. However, these approaches sometimes result in low yields of the desired products. Among other factors, this may arise from the increased pressure to oxidize NADH by other pathways (i.e., ethanol production) and from the expression of alternative *ldh* genes [2, 13]. In this context, genome availability of industrially important microorganisms is of crucial importance in the development of new strategies aimed at strain improvement. In this work, we tried to determine whether the four genes of the *L. casei* BL23 genome assumed to encode lactate dehydrogenases would contribute to lactate synthesis.

A *Δldh1* mutation had a profound effect on the metabolism of *L. casei* but despite the low Ldh activity measured in the mutant strain, it still produced a considerable amount of L-lactate. Two possibilities may explain this: (1) our assay conditions were not optimal for detecting lactate dehydrogenase activity of the additional Ldh enzyme(s) or (2) a very low Ldh activity is enough to give rise to a substantial lactate accumulation in the medium at the end of growth. In *Lactobacillus johnsonii*, inactivation of the gene encoding D-Ldh resulted in more than 85% decrease in Ldh activity [10], whereas total lactate production was only lowered by 16%, suggesting that Ldh enzymes are present in excess for transformation of pyruvate. In any case, the enzyme(s) responsible for residual lactate production in *L. casei* is(are) unknown. The contribution of the newly identified *ldh2*, *ldh3* and *ldh4* to lactate production is low and it is only reflected by small changes in the D-/L-lactate ratio in double mutants. The possibility exists that they encode redundant Ldhs and that deficiency in one gene can be compensated by the additional two genes. This hypothesis could be proven if triple or quadruple mutants in the *ldh* genes would be constructed. Additionally, the presence of extra bands exhibiting Ldh activity in zymogram assays points to the existence of other unidentified enzymes able to reduce pyruvate to lactate.

Our results confirm the previous hypothesis that HicD was responsible for the increased D-lactate levels in the absence of Ldh1 [19]. This effect is totally reverted in a *Δldh1 hicD* double mutant. It appears, therefore, that the HicD enzyme may account for a considerable part of the pyruvate flux towards lactate only when the major Ldh activity is abolished. This probably reflects a lower affinity of HicD for pyruvate compared to Ldh1. By the contrary, affinities for pyruvate of HicD and the additional L-Ldh enzymes might be similar. A search for additional HicD enzymes encoded in the BL23 genome shows a gene coding for a protein with 33% identity to HicD and which is 100% homologous to LSEI_2156, present in the ATCC334 genome [14]. Moreover, zymogram analysis showed a faint band with HicD activity. Whether this putative HicD enzyme plays a role in D-lactate synthesis needs to be proven. In a previous report, mutation of *hicD* resulted in almost undetectable D-lactate levels [19]. However, the present experiments showed that the small production of D-lactate of the wild-type persisted in a *hicD* strain. These differences may be a consequence of the different culture conditions. Similarly, some authors have reported that an *L. plantarum ldhL ldhD* double knockout produced 12–14 mM lactate [3], whereas 60 mM lactate production was reported by other authors from similar amounts of glucose [13].

Although Ldh1 is responsible for the main Ldh activity in *L. casei* BL23, this protein could not be detected by

zymogram analysis. It is thus possible that this enzyme does not migrate in a non-denaturing gel, that it is irreversibly inactivated by SDS or that its activity is lost after gel electrophoresis. However, zymogram analysis confirmed that in *L. casei* BL23 two NADH oxidases are induced by mutation of *ldh1*, which illustrates how cells respond trying to compensate the lack of NADH regeneration via Ldh. The NADH/NAD imbalance resulting from an *ldh1* deletion could be partially compensated by the expression of NADH oxidases which may be $O_2$-dependent and in some LAB account for a substantial proportion of NADH recycling [5]. In fact, *L. casei ldh1* mutants grow faster under aeration [19] and genome inspection of BL23 shows that it encodes at least three putative NADH oxidases (data not shown).

The presence of one additional band exhibiting L-Ldh activity in zymograms proves that the enzymatic machinery for pyruvate reduction in *L. casei* BL23 is more complicated than it was anticipated. Interestingly, as shown with the induced NADH oxidases, this activity was higher upon *ldh1* deletion. In some LAB, growth under aerobiosis induces $O_2$-dependent NADH oxidases [5] and studies on the changes of sugar metabolism in *ldh* knock-out strains [3, 16, 19] have revealed a profound readjustment of the metabolism towards $NAD^+$ regeneration by alternative pathways. The response to *ldh* deficiency in *L. casei* BL23 is complex and it involves not only changes in the pyruvate flux, but also induction of proteins related to pyruvate and NADH metabolism. This stresses the need for a deeper knowledge of the metabolism of these compounds in *L. casei* as the basis for further metabolic engineering approaches.

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