The d-2-Hydroxyacid Dehydrogenase Incorrectly Annotated PanE Is the Sole Reduction System for Branched-Chain 2-Keto Acids in Lactococcus lactis

Emilie Chambellon,1 Liesbeth Rijnen,2‡ Frédérique Lorquet,1§ Christophe Gitton,1 Johan E. T. van Hylckama Vlieg,2 Jeroen A. Wouters,2 and Mireille Yvon1*

INRA, UR 477 Biochimie Bactérienne, F-78350 Jouy-en-Josas, France, ‡ and Department of Flavour, NIZO Food Research B.V., 6710 BA Ede, The Netherlands

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Hydroxyacid dehydrogenases catalyze the stereospecific reduction of branched-chain 2-keto acids to 2-hydroxy acids. These enzymes are used in the production of semisynthetic antibiotics or pharmaceuticals. In this study, we used both targeted and random mutagenesis to identify the genes responsible for the reduction of 2-keto acids derived from amino acids in Lactococcus lactis. The gene panE, whose inactivation suppressed hydroxyisocaproate dehydrogenase activity, was cloned and overexpressed in Escherichia coli, and the recombinant His-tagged fusion protein was purified and characterized. The gene annotated panE was the sole gene responsible for the reduction of the 2-keto acids derived from leucine, isoleucine, and valine, while ldh, encoding L-lactate dehydrogenase, was responsible for the reduction of the 2-keto acids derived from phenylalanine and methionine. The kinetic parameters of the His-tagged PanE showed the highest catalytic efficiencies with 2-ketoisocaproate, 2-ketomethylvalerate, 2-ketoisovalerate, and benzoylformate (Vmax/Km ratios of 6,640, 4,180, 3,300, and 2,050 U/mg/mM, respectively), with NADH as the exclusive coenzyme. For the reverse reaction, the enzyme accepted d-2-hydroxy acids but not l-2-hydroxy acids. Although PanE showed the highest degrees of identity to putative NADP-dependent 2-ketopantoate reductases (KPRs), it did not exhibit KPR activity. Sequence homology analysis revealed that, together with the d-mandelate dehydrogenase of Enterococcus faecium and probably other putative KPRs, PanE belongs to a new family of d-2-hydroxyacid dehydrogenases which is unrelated to the well-described d-2-hydroxyisocaproate dehydrogenase family. Its probable physiological role is to regenerate the NAD+ necessary to catabolize branched-chain amino acids, leading to the production of ATP and aroma compounds.

Hydroxyacid dehydrogenases catalyze the stereospecific and reversible reduction of 2-keto acids to 2-hydroxy acids. These NAD(H)-dependent oxidoreductases are of interest in a variety of fields. Firstly, they are valuable catalysts for the production of the stereospecific isomers of 2-hydroxy acids that are used in the production of semisynthetic antibiotics or pharmaceuticals. Secondly, in lactic acid bacteria, hydroxyacid dehydrogenases are believed to be negatively involved in flavor production, since they compete with other enzymes generating flavor compounds from 2-keto acids derived from amino acids (66), while 2-hydroxy acids are not aroma compounds or precursors of flavor compounds. Indeed, overexpression of the d-2-hydroxyisocaproate dehydrogenase (d-2-HicDH) of Lactobacillus casei has been shown previously to decrease the production of aroma compounds and to delay flavor formation in low-fat cheddar cheese (12). Additionally, hydroxyacid dehydrogenases are involved in the biopreservation properties of lactic acid bacteria, because certain 2-hydroxy acids exhibit antitufungal and antilisterial activities (15, 37, 64). Several hydroxyacid dehydrogenases in lactic acid bacteria have been characterized previously (21, 27). Lactate dehydrogenases (LDH), responsible for the specific reduction of pyruvate to lactic acid, have been studied extensively (2, 7, 13, 16, 21, 40, 49, 54). HicDHs and mandelate dehydrogenases (manDHs) are active toward a broad range of 2-keto acids, including straight-chain aliphatic 2-keto acids, branched-chain 2-keto acids, and 2-keto acids with aromatic side chains, in several lactic acid bacteria have also been characterized previously (5, 6, 27, 28, 29, 38, 39, 50). Although manDHs and HicDHs of lactic acid bacteria prefer 2-ketoisocaproate (KIC) among 2-keto acid substrates, they differ in their activities toward C-3-branched substrates. In particular, manDHs exhibit high levels of activity toward 2-ketoisovalerate (KIV) and benzoylformate, unlike HicDHs. LDHs, HicDHs, and manDHs are divided into two groups, the L- and D groups, depending on the stereoisomer produced. d-LDHs and d-HicDHs are members of the same family of 2-hydroxy acid dehydrogenases, which is distinct from the L-LDH family (13, 54). In general, each lactic acid bacterium contains several hydroxyacid dehydrogenases.

In Lactococcus lactis, which is widely employed as a starter in cheese production, the main LDH is an L-LDH activated by fructose 1,6-bisphosphate (FBP) (23). In addition to the ldh gene that encodes the l-LDH (40), L. lactis contains other genes showing significant levels of similarity to the hydroxyacid dehydrogenases of other lactic acid bacteria (9), but their func-
tions remain unknown, except for that of the recently identified ldhB gene. ldhB is a silent gene that can be activated in ldh-deficient strains via an IS981 element-specific insertion to produce a functional LDH (10). However, L. lactis produces 2-hydroxyacids from the 2-keto acids derived from amino acids not only in vitro, using resting cells, but also in cheese (65, 66). In L. lactis, the catabolism of the aromatic and branched-chain amino acids that are precursors of aroma compounds is initiated by aminotransferases, producing 2-keto acids. These 2-keto acids can be further catabolized to carbonylic acids via a 2-keto acid dehydrogenase, a transacetylase, and a kinase or reduced to 2-hydroxyacids by a hydroxyacid dehydrogenase (66) (Fig. 1). In some strains, branched-chain 2-keto acids can also be decarboxylated to aldehydes that are potent aroma compounds. The aim of the present study was to identify and characterize the enzyme(s) involved in the production of 2-hydroxyacids from the 2-keto acids derived from phenylalanine and branched-chain amino acids and to evaluate its impact on amino acid catabolism.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** The strains used during this study are listed in Table 1. Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) medium. If appropriate, erythromycin, chloramphenicol, ampicillin, or kanamycin was used at a concentration of 150, 10, 50, 10, or 30 μg/ml, respectively. Lactococcal strains were grown at 30°C in M17 broth (Difco, Detroit, MI) or chemically defined medium (52) supplemented with glucose at a final concentration of 0.5% (wt/vol). When necessary, erythromycin, chloramphenicol, or tetracycline was used at a concentration of 5 μg/ml.

**DNA techniques.** All DNA manipulations were performed as described by Sambrook and Russell (48). DNA restriction and modification enzymes were purchased from Invitrogen (Cergy Pontoise, France), Eurogentec (Seraing, Belgium), or Boehringer (Mannheim, Germany) and used as recommended by the suppliers. The oligonucleotides used during this study were synthesized by Eurogentec and are listed in Table S1 in the supplemental material. L. lactis electrocompetent cells were prepared and transformed as described previously by Holo and Nes (25). Plasmid DNA was prepared according to the method described by O’Sullivan and Klennhammer (46). PCR amplifications were carried out using a model 2720 DNA thermal cycler (PerkinElmer, Courtaboeuf, France) by using Taq polymerase (Biogene, Illkirch, France) or Phusion high-fidelity PCR master mix (Finzymes, Finland). Samples for sequencing were prepared using the PRISM ready reaction dye deoxy terminator cycle sequencing kit (Applied Biosystems, Courtaboeuf, France), and the sequences were determined with an ABI PRISM 310 automatic DNA sequencer (Applied Biosystems).

**Inactivation of genes and complementation.** In most mutant strains (Table 1), the gene of interest was disrupted by single-crossover (SCO) integration of a nonreplacable plasmid (pGEM-T Easy or p-Orinewlux) containing an internal fragment of the gene. These internal fragments were obtained by PCR amplification with the primers listed in Table S1 in the supplemental material. LD26 and LD27 for the ygiC gene amplification, LD36 and LD37 for idhB, LD50 and LD51 for ldh, LD58 and LD59 for idhX, and panE2 and panE3 for panE were used. When pGEM-T Easy (Ap') was used, the chloramphenicol resistance determinant (60) or the tetracycline resistance gene of pG plus hostB (26, 44) was cloned into the multiple cloning site because the Ap' marker is not functional in gram-positive bacteria. The tetracycline cassette was recovered from pG plus hostB by Sall/SacII digestion and cloned into the Ncol/SaclI-linearized pGEM-T construct after the treatment of both fragments with T4 DNA polymerase (New England Biolabs, Ipswich, MA) to generate blunt ends. Similarly, the chloramphenicol cassette was recovered from pVE6083 by PstI/EcoRI digestion and cloned into the PstI/SaclI-linearized pGEM-T construct after the treatment of both fragments with T4 DNA polymerase. All these gene inactivations were confirmed by PCR with one primer corresponding to the plasmid used for construction and one primer corresponding to a sequence within the gene but not within the fragment cloned into the plasmid. For the TIL383 strain, the hicD gene was inactivated by double crossover with the thermosensitive plasmid pG pJIM2246 vector (8, 44) containing the complete gene interrupted by chloramphenicol resistance genes (60). The fragment containing the hicD gene was amplified with primers LD32 and LD33. It was digested and cloned into KpnI/EcoRI-linearized pGEM-T2246 vector. The resulting plasmid was produced in E. coli TG1 and used to transform TIL383. The resulting strain was named TIL508(pJIM103pLemoE).

**Identification of the gene responsible for HicDH activity.** The random insertion mutant library previously constructed from L. lactis B1157 (53) by using the thermosensitive plasmid pG plus host9::ISS1 (43, 44) was screened to find a mutant deficient in HicDH activity. The mutants (n = 3,384) were grown overnight in 96-well plates containing M17 medium supplemented with 0.4% lactose and 5 μg/ml erythromycin at 40°C. The cells were harvested, washed twice in 25 mM phosphate buffer (pH 7.5), and disrupted by the Bio 101 FastPrep system (Santan, Hollbrook, NY) twice for 30 s. The protein content was determined using the Bradford method (11), and HicDH activity in a reaction mixture containing 250 mM potassium phosphate buffer, pH 7.5, 0.5 mM NADH, 10 mM 2-ketoscaproic acid, and cell extract in a final volume of 250 μl was measured spectrophotometrically at 340 nm and 30°C for 15 min. The reduction activities of the mutants affected in their abilities to reduce KIC were then precisely determined by the hydroxyacid dehydrogenase assay described below.

**To determine the pG plus host9::ISS1 integration site in the HicDH-negative clone (1296c), total DNA was extracted using a rapid total DNA extraction protocol (24), digested using EcoRI or HindIII, and then ligated using T4 DNA ligase (New England Biolabs). Ligation mixtures were used to transform E. coli TG1-RepA cells. Transformants containing pG plus host9::ISS1 were selected by plating onto LB medium containing 150 μg/ml erythromycin. Plasmids of the transformants were isolated using a Qiagen miniprep kit and sequenced using pG plus host9::ISS1-derived primers. P091 and PGHIS were used for plasmids originating from HindIII digestion, and P092 and PGEcoli were used for plasmids originating from EcoRI digestion.

**Sequence homology analysis.** Homology relationships were established using BLASTP (1). Alignment and phylogenetic analysis were performed with CLUSTAL W (version 1.8) (58).
**Preparation of a crude extract from *L. lactis* strains.** Cells from a 100-ml culture at an optical density at 480 nm of 3 were centrifuged (10 min at 4°C and 14,100 × g) and washed twice with 10 ml of 50 mM triethanolamine (TEA) buffer, pH 7.0. The cells were then suspended in 5 ml of TEA supplemented with 1.6 mg of sucrose/ml and the suspension was incubated for 2 h at 30°C. The spheroplast pellet was harvested by centrifugation (4,100 × g for 15 min at 4°C) and resuspended in hypotonic TEA buffer to provoke spheroplast lysis. Cell debris was eliminated by centrifugation at 20,000 × g for 20 min, and the supernatant was used as a crude extract. The protein content of the crude extract was measured using the Bradford method.

**Recombinant protein production and purification.** The *panE* gene was amplified by PCR from TIL46 total DNA with primers PanE7 and PanE8, containing NdeI and XhoI restriction sites, respectively, and the Phusion high-fidelity PCR master mix (Finnzymes). The amplified fragment, 0.95 kb in length, was digested and cloned into the NdeI/Xhol-linearized pET28a expression vector (Novagen, Gibbstown, NJ), resulting in a N-terminal hexa-His tag fusion. *E. coli* Rosetta cells (Novagen) were transformed with the ligation product, and the transformants were selected on LB medium containing chloramphenicol (10 μg/ml) and kanamycin (30 μg/ml). The plasmid construct pET28a::panE was sequenced to verify that the *panE* sequence was correct. An overnight preculture of 20 ml at 37°C was used to inoculate 1 liter of LB medium containing 10 μg/ml chloramphenicol and 30 μg/ml kanamycin. The culture was grown at 37°C with shaking at 200 rpm. When the culture reached an optical density at 600 nm of 0.6, gene expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and the culture was maintained at 30°C with shaking. Cells were harvested 3 h after induction and suspended in 20 ml of equilibration buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl) containing 10 μM imidazole. Cells were broken using a BASIC Z cell disrupter (Celd, Warwickshire, United Kingdom) at a pressure of 1.6 × 10⁸ Pa. The suspension was centrifuged at 15,000 × g for 20 min to remove unbroken cells and cell debris. The supernatant was placed in contact with 5 ml of nickel-nitritolriacetic acid agarose resin (Qiagen) for 1 h at room temperature. A Poly-Prep column (Bio-Rad) was then packed with the protein-resin complex and washed with 10 volumes of equilibration buffer containing 250 mM imidazole, and 1-ml fractions were collected. Finally, the fractions containing the enzyme were pooled, concentrated by filtration through a Centricon YM10 device (Amicon GmbH, Witten, Germany), and suspended in 100 mM sodium phosphate buffer, pH 7.0. The protein concentration was determined by the Bradford method using bovine serum albumin for calibration.

**Sodium docecyl sulfate-polyacylamide gel electrophoresis** was performed using the minigel system (Novex minicell; Invitrogen) with a 4% Bis-Tris gel. Proteins were visualized by Coomasie brilliant blue staining.

**Molecular mass of the native enzyme.** The apparent molecular mass of the enzyme was determined by gel filtration on a Superdex 200 HR 10/30 column (GE Healthcare, Saclay, France) with 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl.
TABLE 2. Genes of L. lactis IL1403 (NC 002662) and MG1363 (NC 009004) encoding enzymes with the highest degrees of identity to known HicDHs of lactobacilli and impact of their inactivation in TIL46 on HicDH activity

<table>
<thead>
<tr>
<th>IL1403 gene</th>
<th>MG1363 gene (% identity)</th>
<th>Homolog of gene product</th>
<th>Species containing homolog</th>
<th>% Identity of enzymes</th>
<th>HicDH activity in mutant strain (% of activity in wild-type strain)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ldh</td>
<td>ldh (100)</td>
<td>1-HicDH</td>
<td>Lactobacillus confusus</td>
<td>31</td>
<td>193 ± 70</td>
</tr>
<tr>
<td>1114110 (hicD)</td>
<td>Llmg (9475 (88)</td>
<td>1-HicDH</td>
<td>Lactobacillus confusus</td>
<td>41</td>
<td>104 ± 16</td>
</tr>
<tr>
<td>ldhB</td>
<td>ldhB (96)</td>
<td>1-HicDH</td>
<td>Lactobacillus confusus</td>
<td>29</td>
<td>97 ± 17</td>
</tr>
<tr>
<td>ldhX</td>
<td>ldhX (94)</td>
<td>1-HicDH</td>
<td>Lactobacillus confusus</td>
<td>31</td>
<td>128 ± 20</td>
</tr>
<tr>
<td>yugC</td>
<td>Llmg (2242 (97)</td>
<td>1-HicDH</td>
<td>Lactobacillus casei</td>
<td>29</td>
<td>91 ± 14</td>
</tr>
</tbody>
</table>

* Mutant strains were strains with the indicated gene inactivated. Data are means ± standard deviations.

200 mM NaCl and 1 mM dithiothreitol, at a flow rate of 0.3 ml min⁻¹. The gel filtration low- and high-molecular-weight calibration kits were obtained from GE Healthcare.

Hydroxyacid dehydrogenase assay. Reduction activity on 2-keto acids was measured at 37°C with freshly prepared crude extracts from Lactococcus strains or the freshly purified recombinant PanE. The reaction mixture contained 50 mM TEA, pH 7.0, or 100 mM phosphate buffer, pH 7.0, 0.2 mM or 0.3 mM NADH, and 10 mM substrate. 2-Ketopantoate was prepared by hydrolysis of 2-ketopantoyl lactone in 1 mM NaOH (35, 67). The decrease in the absorbance of NADH was monitored at 340 nm (ε = 6.3 mM⁻¹ cm⁻¹). The reduction activities in L. lactis in the absence and presence of 0.2 mM FBP were evaluated. FBP-independent activity is the activity detected in the absence of FBP. The FBP-dependent activity was calculated by subtracting FBP-independent activity from the activity detected in the presence of FBP. The data are means of results from six independent replicate experiments. The conditions for the determination of Kₘ and Vₘₐₓ were those described above, with various substrate concentrations. Experiments were performed in triplicate for each concentration. Kinetic parameters were calculated from double-reciprocal Lineweaver-Burk plots.

The reverse reaction (dehydrogenase activity on 2-hydroxyisocaproate and mandelate) was assayed by coupling NADH formation with a reaction involving the diaphorase which transforms iodonitrotetrazolium into formazan, a red compound. The reaction mixture contained 200 mM TEA phosphate buffer, pH 9.0, supplemented with 1% Triton, 0.6 mM iodonitrotetrazolium, 1 mM NADH, and 10 mM substrate. The formation of red formazan was monitored at 492 nm (ε = 19.4 mM⁻¹ cm⁻¹). All chemicals were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). One unit of enzyme was defined as the amount which catalyzed the oxidation of 1 µmol of NADH or the reduction of 1 µmol of NAD⁺ per min at 37°C.

Amino acid catabolism. The catabolism of leucine was investigated using a reaction medium containing 100 mM potassium phosphate buffer, pH 5.5, 30 mM dithiothreitol (Sigma), 10 mM 2-ketogluaric acid disodium salt (Sigma), 2 mM unlabeled leucine, and 0.05 µM [1-14C]leucine (120 to 190 Ci/mmol; GE Healthcare). The resting cell suspension (0.05 ml) was added to 0.45 ml of the reaction medium, and the mixtures were incubated for 40 h at 37°C. Aliquots (100 µl) of the reaction mixture were collected after 0, 10, 20, and 40 h of incubation, and cells were removed by centrifugation (5,000 × g for 5 min).

The soluble metabolites were then analyzed by reverse-phase high-pressure liquid chromatography and ion exclusion high-pressure liquid chromatography with both UV and radioactivity detection, as described previously (65). Metabolites were identified by comparing the retention times with those of appropriate standard compounds, obtained from Sigma.

RESULTS

Identification of genes involved in the reduction of 2-keto acids. To identify the genes responsible for transforming 2-keto acids into 2-hydroxyacids, two different strategies were applied, one based on targeted mutagenesis and the other based on the selection of randomly generated mutants. In the first strategy, five candidate genes in L. lactis TIL46 encoding enzymes that showed the highest levels of identity to HicDHs in other species of lactic acid bacteria (Table 2) were disrupted separately. The disruption of ldh, hicD, yugC, ldhB, or ldhX did not result in reduced activity with KIC (Table 2). Conversely, the disruption of ldh slightly increased this activity.

Since none of the supposed hic-like genes appeared to be involved in the reduction of branched-chain 2-keto acids, a random mutagenesis strategy was also applied. A random insertion mutant library previously constructed from L. lactis subsp. lactis B1157 by Smit et al. (53) was screened to identify mutants that did not reduce KIC. Only one mutant without HicDH activity in a library of 3,384 mutants was found. In this mutant, the gene inactivated by the insertion of pG+6 host::ISS1 showed 100 and 91% identity to the genes annotated panE in the complete genome sequences of the IL1403 and MG1363 strains, respectively. The panE gene encodes an enzyme of 312 amino acid residues. The highest levels of identity found were those to putative ketopantoate reductases (KPRs; between 24 and 80% amino acid identity), and the enzyme showed only 26 and 24% identity to the known KPRs of E. coli and Salmonella enterica serovar Typhimurium, respectively. In particular, PanE had 74% identity to the putative KPR of Enterococcus faecium that has recently been shown to be a δ-manDH (61). Eighteen of the 21 conserved residues in seven known bacterial KPRs (67) were also conserved in the L. lactis protein, including residues Asn98, Lys176, and Glu256, which are implicated in the catalytic mechanism in E. coli (41, 45, 67), and the GXXGXGXG motif for NAD(P) binding at positions 7 to 12 (see Fig. S1 in the supplemental material). In contrast, the three residues Thr117, Thr119, and His120, which are supposed to play a role in stabilizing ketopantoate (41), were not conserved in the PanE of L. lactis. Surprisingly, the PanE protein showed only 18.4 and 18.7% identity to the δ-HicDH enzymes of Lactobacillus casei and Lactobacillus delbrueckii subsp. bulgaricus, respectively, around 20% identity to the δ-HicDH enzymes of Lactobacillus confusus and Lactobacillus johnsonii, and no significant similarity to the δ-HicDH enzymes of Closstridium difficile (AYY772817). Moreover, none of the catalytically important residues in the enzymes belonging to the family of NAD-dependent δ-2-hydroxyacid dehydrogenases (His296, Arg235, and Glu264) (5, 36, 55, 56) were conserved in PanE. The alignment of the PanE sequence with those of putative KPRs showing between 49 and 80% identity to PanE of L. lactis revealed the presence of 61 additional conserved residues which are not conserved in known KPRs (see Fig. S1 in the supplemental material). No significant similarity between PanE and the NAD-dependent δ-manDH of Rhodotorula gracilis (O14465) was found.
Role of LDH and PanE in the reduction of 2-keto acids by TIL46. The panE gene in the TIL46 strain was disrupted to study the effect of this inactivation on the reduction of 2-keto acids. The effects of the disruption of ldh and panE on the reduction of pyruvate, the main substrate of LDH, and the 2-keto acids derived from leucine (KIC), valine (KIV), isoleucine (2-ketomethylvalerate [KMV]), methionine (2-ketomethylthiobutyrate [KMBA]), and phenylalanine (phenylpyruvate [PPyr]) were tested. Enzymatic assays were performed in the presence or absence of FBP, which is an activator of certain hydroxyacid dehydrogenases (23). FBP-independent and FBP-dependent activity levels are presented in Fig. 2A and B, respectively. The activity of the wild-type strain on pyruvate was exclusively FBP dependent, while the activities of this strain on the branched-chain 2-keto acids (KIC, KIV, and KMV) were exclusively FBP independent. All of these activities were mainly FBP dependent (75% of the total activity), but about 25% of the total activity was FBP independent. All these activities were exclusively NADH dependent. No activity was detected with NADPH. The inactivation of ldh completely abolished FBP-dependent activities on pyruvate, KMBA, and PPyr (Fig. 2B), indicating that LDH is responsible for these activities. In contrast, the inactivation of ldh resulted in an increase in FBP-independent activities on the 2-keto acids derived from leucine, isoleucine, and methionine (Fig. 2A), suggesting an effect on the regulation of FBP-independent activity. This FBP-independent activity was due exclusively to panE since its disruption completely abolished FBP-independent activities on the 2-keto acids derived from the branched-chain amino acids and methionine (Fig. 2A). Moreover, HicDH activity was restored in the complemented mutant strain TIL508 (pJHmpanE), obtained by cloning panE into the multicopy plasmid pJIM2246. Activity in the complemented strain was about 20-fold higher than that in the wild-type strain. In contrast to the effect of ldh inactivation on PanE activity, the inactivation of panE had no effect on the FBP-dependent activities on pyruvate, KMBA, and PPyr, due to LDH.

Characterization of the PanE His-tagged fusion protein. The purified PanE His-tagged fusion protein exhibited a band at around 36 kDa upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. S2 in the supplemental material), which agreed with the calculated mass of the deduced amino acid sequence (34,361 kDa plus 2,163 kDa of His tag peptide). The molecular mass of the recombinant enzyme was estimated to be 70 kDa by gel filtration on a Superdex 200 HR column, indicating that the enzyme is composed of two subunits. The concentrated enzyme solution at pH 7.0 (10.6 mg/ml) could be stored at 4°C for at least 2 to 3 months without significant loss of activity. In contrast, the diluted enzyme solution (4 μg/ml) was not stable and lost 40% of its activity after 1 h at 4°C. Under standard assay conditions, the enzyme exhibited its highest activity at 55°C. At 37°C, the activity reached about 65% of peak activity. The effect of the pH (between pH 5.0 and 8.0) was examined with respect to the reduction of KIC. The optimum pH was about 5.5 to 7.0, and activity was reduced by only about 10% at pH 5.0 and 8.0. At pH 7.0 and 37°C, the specific activities were 2,000 U/mg with KIC and 3,100 U/mg with benzoylformate. The optimum pH for the reverse reaction was about 9.0. The influences of metal ions (Mg²⁺, Ca²⁺, and Cu²⁺), a metal chelator (EDTA), a reducing agent (dithiothreitol), Cys reagents (Hg²⁺ and iodoacetamide), and a His reagent (diethyl pyrocarbonate [DEPC]) (42) on enzyme activity were investigated (Table 3).

The substrate specificity of the recombinant PanE protein was determined according to the kinetics of reduction of a variety of 2-keto acids (Table 4). NADPH did not serve as a cosubstrate with any 2-keto acid, including 2-ketopoatoate. With NADH as a cosubstrate, PanE reduced 2-keto acids with

![FIG. 2. Reduction activities of the L. lactis TIL46 wild type (WT), the ldh mutant, and the panE mutant toward pyruvate, KIC, KIV, KMV, KMBA, and PPyr in the absence (A) or presence (B) of FBP. The data are means ± standard deviations of results for six independent replicates. *, P < 0.01, and **, P < 0.001 (Student’s t test).](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>83 ± 7</td>
</tr>
<tr>
<td>1 mM CaCl₂</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>160 ± 21</td>
</tr>
<tr>
<td>0.1 mM CuSO₄</td>
<td>0</td>
</tr>
<tr>
<td>0.1 mM HgCl₂</td>
<td>0</td>
</tr>
<tr>
<td>0.1 mM iodoacetamide</td>
<td>95 ± 14</td>
</tr>
<tr>
<td>20 mM dithiothreitol</td>
<td>154 ± 5</td>
</tr>
<tr>
<td>1 mM DEPC</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>2 mM DEPC</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>

*Activities were determined with 10 mM 2-ketoisocaproate and 3 mM NADH in 100 mM phosphate buffer, pH 7.0, at 37°C. Values are given as mean percentages ± standard deviations of the activity in the control sample.
showed that, in addition to ldh, which encodes L-LDH (40), *L. lactis* possesses several genes for enzymes showing significant identity to the HicDHs that catalyze the reduction of various 2-keto acids in other lactic acid bacteria. However, the inactivation of these genes suggested that they were not involved in 2-keto acid reduction in *L. lactis*, except for *ldh*, which was responsible for the FBP-dependent reduction of the 2-keto acids derived from phenylalanine and methionine. Similarly, LDHs of other lactic acid bacteria exhibited some activity toward *PPyr* (30, 49, 54, 59).

Random mutagenesis revealed that the activity toward KIC in *L. lactis* was encoded exclusively by a gene that had been annotated *panE* because of the homology of the corresponding protein sequence to those of KPRs, which catalyze the NADPH-dependent reduction of 2-ketopantoate to D-pantoate in the biosynthesis of pantothenate (vitamin B₅). However, although the PanE of *L. lactis* shows the highest degrees of identity to putative KPRs and possesses most of the important residues for KPR activity, it did not exhibit KPR activity; it was not active toward 2-ketopantoate and did not utilize NADPH as a cofactor, unlike known KPRs (18, 51, 68, 69). In many bacteria, a gene has been predicted to encode a KPR on the basis of sequence homology, even if the identity of the corresponding amino acid sequence to those of known KPRs is low (<30%), but for most of these genes, this function has never been confirmed. The KPR functions of only the PanE proteins in *E. coli* and *Salmonella serovar Typhimurium*, which show 87% identity, and PanE of *Bacillus subtilis* (with 28% identity to PanE of *E. coli*) have been demonstrated previously (4, 19). Recently, the gene predicted to encode a KPR in *E. faecium* has been shown to encode a D-panDH (61). This enzyme has 74% identity to the PanE of *L. lactis*.

The kinetic parameters of the purified recombinant His-tagged PanE showed that its substrate specificity was very similar to that of the D-panDH2 from *Enterococcus* and differed from the specificity of known D-HicDHs (see Table S2 in the supplemental material) (34). In particular, like the D-panDHs of *Lactobacillus curvatus* (29) and *Enterococcus faecalis*, PanE exhibited a high level of activity toward C-3-branched substrates such as KIV, KMV, and benzoylformate.
while D-HicDHs hardly utilize them. Also, it exhibited lower activity on straight-chain aliphatic substrates than D-HicDHs. Its optimum pH and temperature, dimeric structure, stability during storage at 4°C, and total inhibition by Cu2+ and Hg2+ were also very similar to the characteristics of the D-manDHs of *E. faecalis* and *Lactobacillus curvatus*, but these characteristics do not clearly distinguish D-manDHs from D-HicDHs. The highest specific activity of the recombinant PanE was that in the presence of benzoylformate (3,100 U/mg), and this activity was five times higher than the activity of the recombinant D-manDH2 of *Enterococcus* (680 U/mg) and close to the activity of the D-manDH of *Lactobacillus curvatus* (2,000 U/mg).

The amino acid sequence of the D-manDH of *Lactobacillus curvatus* is still unknown, and the genome is not available. However, the amino acid sequences of both the D-manDH2 of *E. faecium* and the PanE of *L. lactis* differ completely from those of known D-HicDHs, and none of the catalytically important residues for known D-HicDHs were conserved in PanE and D-manDH2.

All these results indicate that the PanE of *L. lactis* and the D-manDH2 of *E. faecium* belong to a new family of D-hydroxyacid dehydrogenases, distinct from the known D-HicDH family and also from the NAD-dependent D-manDHs found in yeast. The latter are highly specific for d-mandelate and did not show significant similarity to PanE (17). This new family may include other putative KPRs such as those of *E. faecalis*, *Lactobacillus casei*, *Lactobacillus sakei*, *C. perfringens*, and *Streptococcus pneumoniae*, which all show more than 49% identity to *L. lactis* PanE. However, as in HicDHs, a His residue is probably involved in the active site, since activity was inhibited by DEPC treatment. The rapid inactivation of PanE by merciric ions and the stimulation of enzyme activity by dithiothreitol suggested that a Cys residue was also involved in the active center of the enzyme, but this was not confirmed by enzyme preincubation with iodoacetamide, as this treatment did not affect enzyme activity.

Although D-HicDHs and D-manDHs from lactic acid bacteria have been the subject of extensive study for biotechnology applications, their roles in metabolism remain unknown. However, based on their substrate specificities, we can suppose that the production of isovalerate by TIL46 was slightly decreased by panE inactivation. These results suggest that KIC reduction participated to some extent in NADH oxidation and consequently slightly stimulated isovalerate production. We can therefore suppose that the impact of HicDH activity on branched-chain amino acid catabolism will depend on the presence or absence of an electron acceptor in the medium for NAD+ regeneration. In the absence of an electron acceptor, the balance between keto acid dehydrogenase activity and HicDH activity should be favorable to leucine conversion to isovalerate. In contrast, in the presence of suitable electron acceptors, HicDH may compete with the keto acid dehydrogenase activity and HicDH activity should be favorable to leucine conversion to isovalerate.

In conclusion, the present work enabled the discovery in *L. lactis* of a D-hydroxyacid dehydrogenase similar to the D-manDH2 found in *Enterococcus*. These enzymes belong to a new family of D-hydroxyacid dehydrogenases which differs from the D-HicDH family. This is the sole enzyme responsible for the reduction of branched-chain 2-keto acids in *L. lactis*. In the absence of a suitable electron acceptor in the medium, this enzyme can serve to regenerate the NAD+ necessary for the catabolism of branched-chain amino acids, leading to the production of ATP and aroma compounds. Because this enzyme has no KPR activity and prefers 2-keto acid substrates with a branched chain but is not specific for D-mandelate, the encoding gene, previously annotated panE, should be renamed *hdhD* for D-hydroxyacid dehydrogenase.

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