Supplemental Material for:

The Relative Catalytic Efficiency of *ldh*L- and *ldh*D-Encoded Products is Crucial for the Optical Purity of Lactic Acid Produced by *Lactobacillus* Strains

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SUPPLEMENTARY METHODS

Batch fermentation. To determine the exact optical purity of lactic acid produced by the 3 representative *Lactobacillus* strains, *L. bulgaricus* ATCC 11842, *L. plantarum* ATCC 14917, and *L. casei* ATCC 334, batch fermentation was performed in 100-ml flasks containing 50 ml of MRS media with 1% $CaCO_3$ as neutralizing agent. The cultures were incubated at 37°C for 24 h without agitation. Thereafter, the concentration and optical purity of lactic acid were analyzed by high-performance liquid chromatography (HPLC).

L-nLDH and D-nLDH assays. The reduction activities of purified L- and D-nLDHs on pyruvate were assayed at 37°C in 1 ml of 50 mM Tris-HCl buffer (pH 7.0), 0.2 mM NADH, 5 mM fructose 1,6-bisphosphate (FDP), 10 mM $MnSO_4$, appropriate concentrations of pyruvate and the enzyme. The rate of NADH oxidation was determined by measuring the absorbance change at 340 nm. One unit of L- or D-nLDHs was defined as the amount that catalyzed the oxidation of 1 μmol NADH per minute (14). For the determination of L- and D-nLDHs in whole cell extracts of *Lactobacillus* strains, cells in the middle of the exponential phase were collected by centrifugation, washed with 0.85% (w/v) sodium chloride solution, subsequently suspended in 50 mM Tris-HCl buffer (pH 7.0) and then disrupted by sonication. The reduction activities of L- and D-nLDHs were assayed in a reaction mixture containing 20 mM pyruvate, 20 mM NADH and 0.1 mg ml^{-1} whole cell extracts at 37°C for 10 min. Thereafter, activities of nLDHs were inactivated by boiling for 5 min. Then, L- and D-lactic acid was detected by HPLC with a chiral column. Activities of L- and

D-nLDHs in *Lactobacillus* strains were calculated by the corresponding concentrations of L- and D-lactic acid.

Analytical procedures. Lactic acid concentration was measured by HPLC (Agilent 1100 series, Hewlett-Packard, USA) equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and a refractive index detector. Analysis was performed with a mobile phase of 10 mM H_2SO_4 at a flow rate of 0.4 ml min^{-1} at 55°C. Stereoselective assays of L-lactic acid and D-lactic acid were performed by HPLC equipped with a chiral column (MCI GEL CRS10W, Japan) and a tunable UV detector at 254 nm. The mobile phase was 2 mM $CuSO_4$ at a flow rate of 0.5 ml min^{-1} and at 25°C. The optical purity of L-lactic acid was described as enantiomeric excess (ee) value which was defined as $\frac{(\text{L-lactic acid})-(\text{D-lactic acid})}{(\text{L-lactic acid})+(\text{D-lactic acid})}\times 100\%$. Similarly, the optical purity of D-lactic acid was also described as ee value which was defined as $\frac{(\text{D-lactic acid})-(\text{L-lactic acid})}{(\text{D-lactic acid})+(\text{L-lactic acid})}\times 100\%$.

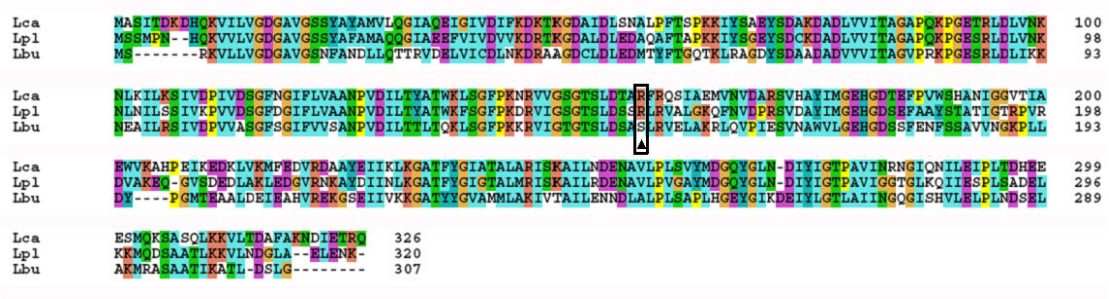

FIG. S1. Multiple alignment of amino acid sequences of the cloned L-nLDHs. Species and accession numbers of sequences were shown in Table S2. Lbu, *L. bulgaricus* ATCC 11842; Lpl, *L. plantarum* ATCC 14917; Lca, *L. casei* ATCC 334. Symbols represent: ▲, Arg171.

There is considerable sequence identity between L-nLDHs of different types of *Lactobacillus* stains (L-nLDHs of *L. bulgaricus* ATCC 11842 and *L. plantarum* ATCC 14917, 53%; L-nLDHs of *L. bulgaricus* ATCC 11842 and *L. casei* ATCC 334, 50%; L-nLDHs of *L. plantarum* ATCC 14917 and *L. casei* ATCC 334, 69%). The crystal structure of L-nLDHs had been solved and residues responsible for substrate binding and catalysis had been identified in previous reports (2, 6, 7, 12). The results revealed that Arg171 is a crucial residue at the substrate-binding site and promotes pyruvate binding (4, 5, 6). But for L-nLDH of *L. bulgaricus* ATCC 11842, Arg171 was substituted by Ser, which suppressed its activity inevitably.

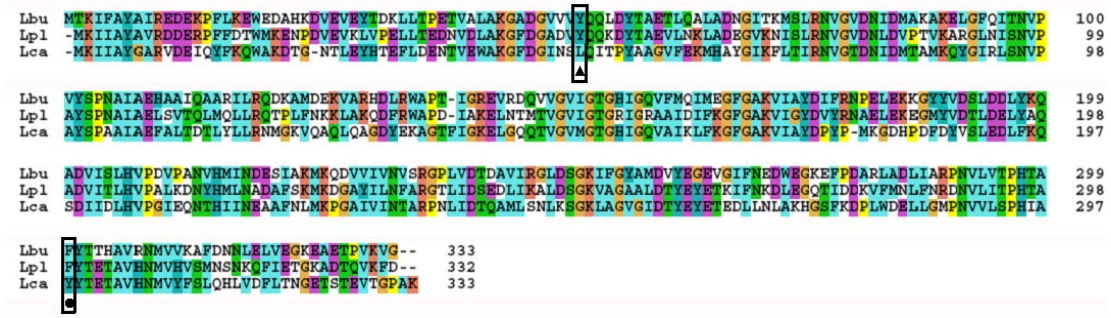

FIG. S2. Multiple alignment of amino acid sequences of the cloned D-nLDHs. Species and accession numbers of sequences were shown in Table S2. Lbu, *L. bulgaricus* ATCC 11842; Lpl, *L. plantarum* ATCC 14917; Lca, *L. casei* ATCC 334. Symbols represent: ▲, Tyr52; ●, Phe299.

There is considerable sequence identity between D-nLDHs of different types of *Lactobacillus* strains (D-nLDHs of *L. bulgaricus* ATCC 11842 and *L. plantarum* ATCC 14917, 53%; D-nLDHs of *L. bulgaricus* ATCC 11842 and *L. casei* ATCC 334, 39%; D-nLDHs of *L. plantarum* ATCC 14917 and *L. casei* ATCC 334, 44%). The crystal structure of D-nLDHs had been solved and residues responsible for substrate binding and catalysis had been identified in previous reports (9, 11, 12, 13, 15). Tyr52 and Phe299 are close to the methyl group of pyruvate. Although they are not as important as Arg171 of L-nLDH for pyruvate binding, they influence the shape of hydrophobic pocket for methyl group of pyruvate and thus are helpful for pyruvate binding (3, 16). In D-nLDH of *L. casei* ATCC 334, Tyr52 and Phe299 were substituted by Leu and Tyr, respectively. This would bring negative effect on its catalytic efficiency as previous reports (8, 16).

88 TABLE S1. Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Source or reference
E. coli strain		
E. coli DH5α	φ80 *lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *recA*1 *endA*1 *hsdR*17 *supE*44λ- *thi*-1	Invitrogen Life Technologies
E. coli Rosetta(DE3)	F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm lacY1* (DE3) pRARE(argU, argW, ileX, glyT, leuW, proL)(Cm^r)	Novagen
Lactobacillus strain		
L. bulgaricus ATCC 11842	Wild-type D-lactic acid producing strain	ATCC
L. plantarum ATCC 14917	Wild-type DL-lactic acid producing strain	ATCC
L. casei ATCC 334	Wild-type L-lactic acid producing strain	ATCC
Plasmid		
pETDuet-1	Expression vector, Amp^r	Novagen
pETDuet-11842*ldh*L	N-terminal His-tagged *ldh*L of *L. bulgaricus* ATCC 11842 in pETDuet-1	This study
pETDuet-11842*ldh*D	N-terminal His-tagged *ldh*D of *L. bulgaricus* ATCC 11842 in pETDuet-1	This study
pETDuet-14917*ldh*L	N-terminal His-tagged *ldh*L of *L. plantarum* ATCC 14917 in pETDuet-1	This study
pETDuet-14917*ldh*D	N-terminal His-tagged *ldh*D of *L. plantarum* ATCC 14917 in pETDuet-1	This study
pETDuet-334*ldh*L	N-terminal His-tagged *ldh*L of *L. casei* ATCC 334 in pETDuet-1	This study
pETDuet-334*ldh*D	N-terminal His-tagged *ldh*D of *L. casei* ATCC 334 in pETDuet-1	This study
Oligonucleotide primer	Sequence (5′→3′) and properties[a]	
q11842ldhL.f	TGACAGAAGCAGCCTTAGATG	
q11842ldhL.r	GCCACGCCATAGTAGGTTG	
q11842ldhD.f	AAGATGAGCCTGCGTAAC	
q11842ldhD.r	TTCGTCCATAGCCTTGTC	
q14917ldhL.f	ATCCTCGTTCCGTTGATG	
q14917ldhL.r	AAGTTGATGATGTCGTAAGC	
q14917ldhD.f	TGGTGTTATCGGTACTGGTC	
q14917ldhD.r	TGTGGTAGTTATCCTTCAATGC	

q334ldhL.f	GCCGTTGGTTCAAGTTATGC
q334ldhL.r	GCGTTGCTCAAGTCAATCG
q334ldhD.f	CCGAGGACTTGTTGAATC
q334ldhD.r	GGTGTAGTAGGCAATGTG
11842ldhL.f	GCGAAGCTTATGAAAAAGGTCAATCGTAT (*Hin*dIII)
11842ldhL.r	TATCTCGAGTCCTAAAGAGTCCAGGGTTG (*Xho*I)
11842ldhD.f	CGCCTGCAGATGACTAAAATTTTTGCTTA (*Pst*I)
11842ldhD.r	TATCTCGAGGCCAACCTTAACTGGAGTTT (*Xho*I)
14917ldhL.f	TATCTGCAGATGTCAAGCATGCCAAATCA (*Pst*I)
14917ldhL.r	GCGCTCGAGTTTATTTTCTAATTCAGCTA (*Xho*I)
14917ldhD.f	CGCAAGCTTATGAAAATTATTGCATATGC (*Hin*dIII)
14917ldhD.r	TATCTCGAGGTCAAACTTAACTTGTGTGT (*Xho*I)
334ldhL.f	GTGCTGCAGGTGGCAAGTATTACGGATAA (*Pst*I)
334ldhL.r	TGTCTCGAGCTGACGAGTTTCGATGTCAT (*Xho*I)
334ldhD.f	CGCAAGCTTATGAAGATTATTGCTTACGG (*Hin*dIII)
334ldhD.r	TATCTCGAGCTTTGCTGGACCAGTAACTT (*Xho*I)

[a]For protein expression, oligonucleotides were designed to introduce recognition sites for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses).

92 TABLE S2. List of sequences included in the alignment and phylogenetic analysis

Assignment	Organisms and relevant type[a]	Accession number	Reference
L-nLDH	*L. bulgaricus* ATCC 11842 (D)	CAI96961	NCBI prokaryotic genome database
L-nLDH	*L. bulgaricus* ATCC BAA-365 (D)	ABJ57783	NCBI prokaryotic genome database
L-nLDH	*L. jensenii* 1153 (D)	EEQ68605	NCBI prokaryotic genome database
L-nLDH	*L. jensenii* 269-3 (D)	EEQ24781	NCBI prokaryotic genome database
L-nLDH	*L. plantarum* ATCC 14917 (DL)	EFK28653	NCBI prokaryotic genome database
L-nLDH	*L. plantarum* JDM1 (DL)	ACT61325	NCBI prokaryotic genome database
L-nLDH	*L. pentosus* DSM 20314 (DL)	BAA14353	14
L-nLDH	*L. fermentum* IFO 3956 (DL)	BAG27142	NCBI prokaryotic genome database
L-nLDH	*L. fermentum* CECT 5716 (DL)	ADJ41146	NCBI prokaryotic genome database
L-nLDH	*L. reuteri* CF48-3A (DL)	EEI65172	NCBI prokaryotic genome database
L-nLDH	*L. buchneri* ATCC 11577 (DL)	EEI19802	NCBI prokaryotic genome database
L-nLDH	*L. brevis* ATCC 27305 (DL)	EEI71300	NCBI prokaryotic genome database
L-nLDH	*L. rhamnosus* Lc 705 (L)	CAR91366	NCBI prokaryotic genome database
L-nLDH	*L. rhamnosus* GG (L)	CAR88418	NCBI prokaryotic genome database
L-nLDH	*L. casei* ATCC 334 (L)	ABJ71272	NCBI prokaryotic genome database
L-nLDH	*L. casei* BL23 (L)	CAQ67767	NCBI prokaryotic genome database
L-nLDH	*X. cellulosilytica* DSM 15894	ACZ30288	NCBI prokaryotic genome database
D-nLDH	*L. bulgaricus* ATCC 11842 (D)	CAI96942	NCBI prokaryotic genome database
D-nLDH	*L. bulgaricus* ATCC BAA-365 (D)	ABJ57770	NCBI prokaryotic genome database
D-nLDH	*L. jensenii* 1153 (D)	EEQ68030	NCBI prokaryotic genome database
D-nLDH	*L. jensenii* 269-3 (D)	EEQ23913	NCBI prokaryotic genome database
D-nLDH	*L. plantarum* ATCC 14917 (DL)	EFK29428	NCBI prokaryotic genome database
D-nLDH	*L. plantarum* JDM1 (DL)	ACT62606	NCBI prokaryotic genome database
D-nLDH	*L. pentosus* DSM 20314 (DL)	BAA14352	14
D-nLDH	*L. fermentum* IFO 3956 (DL)	BAG26968	NCBI prokaryotic genome database
D-nLDH	*L. fermentum* CECT 5716 (DL)	ADJ41057	NCBI prokaryotic genome database
D-nLDH	*L. reuteri* CF48-3A (DL)	EEI64675	NCBI prokaryotic genome database
D-nLDH	*L. buchneri* ATCC 11577 (DL)	EEI18861	NCBI prokaryotic genome database
D-nLDH	*L. brevis* ATCC 27305 (DL)	EEI69958	NCBI prokaryotic genome database
D-nLDH	*L. rhamnosus* Lc 705 (L)	CAR88994	NCBI prokaryotic genome database
D-nLDH	*L. rhamnosus* GG (L)	CAR86053	NCBI prokaryotic genome database
D-nLDH	*L. casei* ATCC 334 (L)	ABJ69009	NCBI prokaryotic genome database

D-nLDH	*L. casei* BL23 (L)	CAQ65269	NCBI prokaryotic genome database
D-nLDH	*E. coli* MG1655	NP_415898	NCBI prokaryotic genome database

[a]LAB, including *Lactobacillus* genus, were classified into 3 types (L-LAB, DL-LAB, and D-LAB) based on the stereoisomer of lactic acid. L-LAB means the main product is L-lactic acid; DL-LAB means the main product is DL-lactic acid; D-LAB means the main product is D-lactic acid. All the selected *Lactobacillus* strains were frequently used lactic acid producers and their relevant types were in accordance with the previous reports (1, 10).

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